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Award Number: DAMD17-01-1-0688

TITLE: Novel Tissue Models of Junctional Epidermolysis Bullosa to Characterize Functional Mechanisms of Sulfur Mustard Injury to Human skin

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REPORT DATE: March 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 01-03-2005 | Final | 1 May 2001 –28 Feb 2005 |

**4. TITLE AND SUBTITLE**
Novel Tissue Models of Junctional Epidermolysis Bullosa to Characterize Functional Mechanisms of Sulfur Mustard Injury to Human skin

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
DAMD17-01-1-0688

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Jonathan Garlick, D.D.S., Ph.D.

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

The Research Foundation of SUNY
Stony Brook, NY 11794

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

This final report is essentially the same as the third year report. We first measured dose-time responses in 2-D and 3-D cultures (TASK 1) and discovered that an SM dose of 150uM induced significant apoptotic cell death. We next compared the SM response of 3-D cultures grown in the absence or presence (AlloDerm) of structured basement membrane (BM) (TASK 8) and found that the presence of BM led to resistance to SM-induced damage, suggesting that BM could protect basal keratinocytes from SM-induced apoptosis. To further explore the role of BM in decreased SM susceptibility, primary keratinocytes harvested from Junctional Epidermolysis Bullosa (JEB) patients (#552), that lack a functional $\gamma 2$ chain of laminin 5 and do not adhere to BM, were transduced with retroviral vectors (TASK 4) to restore or abrogate laminin 5-mediated adhesion. We constructed 3-D tissues with these "reverted" JEB cells (TASK 5,6 and 7) and their phenotypic analysis showed that only JEB cells with restored laminin 5 function (F-GAL) were resistant to apoptosis when exposed to SM (150uM), thereby implicating laminin 5-mediated attachment as being important in limiting SM damage. These studies provide important evidence that bioengineered, *in vitro* tissues mimic many skin alterations previously found *in vivo* and that adhesion to BM enables epithelial resistance to SM damage.

**15. SUBJECT TERMS**
Sulfur mustard, basement membrane, organotypic culture, keratinocytes

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | USAMRMC |
| U | U | U | UU | 60 | 19b. TELEPHONE NUMBER *(include area code)* |

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## INTRODUCTION

A major reason for our limited understanding of what triggers SM injury is that detailed mechanistic studies using human skin have not been possible for ethical reasons. Therefore, we have used an approach that has allowed us to identify sites and pathways of sulfur mustard (SM)-induced vesication using engineered human skin that mimics the clinical and histologic features of this tissue. Through research conducted this past year, our laboratory has extensively studied the pathophysiology of bioengineered, *in vitro,* 3-D human skin in response to SM by establishing dose/time responses of these human, skin-like tissues that lead to dermal-epidermal separation. We have developed and adapted novel tissue models that have found that structured basement membrane can alter the response to SM injury by making the tissue less susceptible to SM-mediated damage. We used immunohistochemical, morphologic and biochemical analyses to characterize the influence of these ECM and BM subtrates on the morphogenesis, survival, differentiation and growth of NHK. We have found that the presence of individual BM components, Type IV and tissues grown on intact complete BM (de-epidermalized dermis) in organotypic cultures supports the survival of these skin-like tissues. Since there is a significant linkage between the pathologic alterations in Junctional Epidermolysis Bullosa (JEB) and SM injury, our understanding of he molecular defects in in BM causing JEB should shed light on the pathogenesis of SM-induced blistering. To further explore the role of basement membrane proteins in keratinocyte resistance to SM-induced damage, we directly studied the role of laminin 5 in this process. Primary keratinocytes harvested from patients with the blistering skin disease Junctional Epidermolysis Bullosa (JEB), that lack a functional gamma 2 chain of laminin 5 and are not able to adhere to basement membrane, were transduced with retroviral vectors designed to restore laminin 5-mediated adhesion. We found that only JEB cells in which laminin 5 adhesive function was restored (F-GAL) were resistant to apoptosis when exposed to SM (150 ug/ml), thereby implicating laminin 5-mediated attachment as being important in limiting SM damage. These studies provide important proof of concept that *in vitro* and *in vivo* tissue models mimic many of the tissue alterations previously found in animal models of SM injury. Our findings show that adhesion to basement membrane proteins enables subsets of keratinocytes to resist SM damage. Since we have previously found that only specific subsets of basal keratinocytes underwent cellular damage leading to apoptosis in our *in vivo* engineered tissue models, we have taken an important step towards defining the ECM or BM components that provide survival signals that can protect cells when challenged with SM. These human tissue models will be of great relevance in understanding functional mechanisms of SM injury and in testing new countermeasures to limit its morbidity and mortality.

## EXPERIMENTAL RESULTS:

# PART I: ESTABLISHMENT OF CONDITIONS UNDER WHICH SULFUR MUSTARD CAN ALTER THE SURVIVAL AND VIABILITY OF NORMAL HUMAN KERATINOCYTES AND JUNCTIONAL EPIDERMOLYSIS BULLOSA CELLS GROWN ON A VARIETY OF BASEMENT MEMBRANE COMPONENTS IN 2-D MONOLAYER CULTURES

***TASK 1: To determine the dose/time responses of normal keratinocytes to sulfur mustard exposure***

**1. Determination of the effects of ethanol on human keratinocytes:** Prior to the application of sulfur mustard (SM) on cultured cells, we conducted a series of experiments using various concentrations of pure ethanol to determine the effects of this solvent on keratinocyte growth and survival. This was done because SM was dissolved in pure ethanol and it was important to determine if this vehicle would induce alterations in cultured cells at the SM concentrations needed. Experiments were carried out in p60 plates and keratinocytes were grown on feeder layers of γ-irradiated 3T3 cells until colonies were 60% confluent. Cultures were then exposed to different doses of ethanol (0.4%, 0.8%, 1.6%) for 30 min and compared to untreated controls. Plates were then rinsed three times with fresh media and were grown for an additional week. Colonies were stained using crystal violet and the diameter of colonies exposed to ethanol doses were measured. ***Based on our experiments we concluded that ethanol did not greatly alter the growth of keratinocytes compared to the non-exposed controls as the size of individual colonies was not altered upon exposure to ethanol (Figure 1).***

**2. Staining for cellular apoptosis to determine the dose and time response of cultured keratinocytes to sulfur mustard:** To establish the SM doses that could induce alterations in keratinocyte growth, a dose and time study was performed to measure the response of human keratinocytes to SM. Keratinocytes were seeded on sterile coverslips with 3T3 feeder layers (10,000 cells/p60) for six days in p60 plates until the colonies became 70% confluent. Cultures were then exposed to different doses of SM and compared to ethanol-exposed controls. We initially selected four different doses 37.5, 75, 150, 300μM of SM and compared these to 0.25, 0.5, 1, 2% ethanol upon exposure for 7 min. Cells on coverslips that were exposed to agents were then processed 24 hours later for immunofluorescent staining for the presence of apoptotic cells using a monoclonal antibody that detects the cleavage product of keratin 18 that is the end result of apoptotic pathways (M30 Cytodeath-stain, Roche, Inc.). Fig. 3 shows the appearance of apoptotic cells that demonstrated M30 staining in the cytoplasm (red stain) for different doses of SM when compared to corresponding ethanol controls. At low SM doses of (37.5μM, 75μM), fewer apoptotic cells were seen when compared to 150 and 300μM SM doses. All doses of ethanol showed fewer apoptotic cells than SM-exposed cells. ***Thus a dose-dependent increase in the number of apoptotic cells was seen after SM treatment. Based on these findings, we selected 150μM as a standard SM dose that induced a maximum number of apoptotic cells.***

An additional experiment was performed to study the effect of the length of exposure to SM on cell survival and death. We selected a dose of 150μM to study the effects of SM exposure for 1,3, 7, 14, and 28 min. Staining was performed for apoptotic keratinocytes grown on coverslips using the M30 stain the numbers of apoptotic cells on 3 coverslips were counted for each length of exposure. A sharp increase in the number of apoptotic cells was seen for 7 min exposures when compared to 1 and 3 min exposures. Longer exposures (14 min and 28 min) resulted in an 8–10 fold increase in numbers of apoptotic cells when compared to cultures exposed for 7 min (Fig. 5). In comparison, untreated controls showed a very small number of apoptotic cells while ethanol showed very small changes in the number of apoptotic cells. Fig. 6 demonstrates the appearance M30 positive cell after exposure to SM and to 1% ethanol for 7 min. ***These findings demonstrated that a SM dose of 150μM at an exposure time of 7 min was sufficient to induce significant apoptotic cell damage in 2-D cultures of keratinocytes. As a result, it was decided to use 7 min as the standard SM exposure time for all future studies.***

**3. MTT viability/survival assays to determine keratinocyte survival after SM exposure:** We next performed the MTT assay to evaluate the viability of normal keratinocytes after exposure to SM. In this assay, the yellow tetrazolium salt MTT is reduced in metabolically active cells to form insoluble purple formazan crystals that are solubilized by the addition of a detergent. The purple color can then be quantified by spectrophotometric means and provides a direct measure of cell viability upon exposure to SM. Conversely, a reduction in spectrophotometric measurement reflects the loss of cell viability. We performed MTT assays using normal human keratinocytes (NHK) at different cell densities to establish if varying cell density could alter cell viability in response to SM. NHK's were plated at densities of $5X10^4$, $2.5X10^4$, $1X10^4$ and $1X10^3$ and exposed to either 150μM of SM or to 1% ethanol control. Fig. 7 demonstrates that cells seeded at low densities (1,000 and 10,000 cells) showed no difference in cell viability when SM exposures were compared to ethanol controls (Fig. 7). However, at high cell densities (25,000 and 50,000 cells), SM exposure significantly decreased cell viability when compared to ethanol-exposed controls. ***This demonstrated that the sensitivity of detection of MTT assay required a threshold number of cells greater than 25,000 to yield differences in cell survival between SM- and ethanol-exposed cells. These studies laid the groundwork for all experiments by establishing parameters required for length and concentration of SM exposure needed to alter cell viability and to induce apoptosis.***

***TASK 8 : Dose-time response to establish the role of basement membrane components to SM in 2-D cultures***

**4. The role of basement membrane components on the survival and viability of keratinocytes exposed to sulfur mustard** - We next performed experiments to establish the importance of (BM) or extracellular matrix components (EMC) on cell viability and cell survival when exposed to different doses of SM compared to ethanol controls. To establish if the basement membrane (BM) protein Type IV collagen could alter the sensitivity of normal keratinocytes to SM, we seeded different numbers of keratinocytes on Type IV Collagen-coated, 24-well plates and performed MTT viability assays (Fig.8). Results were similar to those seen when cells were plated on tissue culture plastic as SM-exposed cells showed lower viability when compared to those ethanol-exposed at higher cell density (50,000 and 25,000). Similar differences were seen when cells were exposed to SM and ethanol after seeding onto Type I Collagen coated plates (Fig. 9). These experiments demonstrated that an SM dose of 150μM for 7 min reduced cell survival compared to ethanol exposure. Fig.10 presents MTT viability assays when 50,000 cells were plated on different substrates including plastic, Type I Collagen, Type IV Collagen, Fibronectin, Laminin and Poly D-lysine in monolayer, 2-D cultures. 24 hours after seeding, cells were exposed to 150μM SM or 1% ethanol for 7min. All the plates showed a decrease in cell viability when treated with SM compared to ethanol controls. However, the greatest reduction in viability (50%) upon SM exposure was seen for cells grown on Type IV Collagen. Since cells grown on this substrate also demonstrated the highest cell viability when exposed to ethanol, it is possible that the elevated cell growth on Type IV Collagen made cells more vulnerable to SM damage. Other substrates (Type I Collagen, Fibronectin and Laminin) demonstrated decreased viability upon SM exposure, but to a lesser degree than Type IV Collagen. Poly-D-lysine and plastic-coated dishes demonstrated the smallest loss of viability upon SM exposure. This may be due to the poor attachment of keratinocytes to poly D-lysine-coated plates compared to other substrates.

MTT assays were carried out for varied doses of SM and ethanol on different substrates (Fig. 11). Four different doses of SM were tested (37.5, 75, 150, 300μM) and were compared to controls (0.25, 0.5, 1, 2% ethanol) when cells were seeded onto tissue culture plastic, Type I Collagen, Type IV Collagen, Fibronectin and Laminin. As described above, the greatest decrease in viability after SM exposure was seen for cells grown on Type IV Collagen. Interestingly, this was the only substrate that demonstrated loss of cell survival even at low SM doses (37.5μM and 75μM). Other substrates showed no loss of viability for these low SM doses but did show a moderate decrease at higher SM doses. Thus, while ECM components were protective at low SM doses, they did not provide a survival advantage at higher SM doses. ***Significantly, cells grown on the BM component Type IV Collagen demonstrated the***

***greatest sensitivity of cells to SM-induced damage in 2-D cultures. These findings demonstrated that individual ECM or BM components were not able to provide protection from SM damage in 2-D cultures and intact BM may be required to mediate this event.***

***TASK 5: Dose-time response to establish the response of JEB cells to low dose SM exposure***

**5. <u>MTT Assay to determine the response of Junctional Epidermolysis Bulllosa (JEB) cells to SM:</u>**
There is a significant linkage between the pathologic alterations seen in Junctional Epidermolysis Bullosa (JEB) and those seen in vesicant injury induced by SM. To determine if cells lacking the ability to synthesize a functional laminin 5 molecule would demonstrate an altered sensitivity to SM exposure, we utilized primary keratinocytes that were derived from patients with JEB that were deficient in laminin 5 function. JEB cell lines were initially harvested from the skin of a patient with JEB (552) by the laboratory of Dr. Guerrino Meneguzzi (INSERM, Nice, France). Cells were infected with retroviral vectors that were previously shown in our lab (Progress Report 1) to modify laminin 5 function and restore cell-substrate adhesion in cells that were adhesion-deficient due to the absence of Gamma 2 chain (Phoenix producer cells courtesy of Nolan lab). The following vectors were used to modify laminin 5 function in 552 cells:

**Delta BC:** Cleaved variant of Gamma 2 chain of laminin 5 with a shortened 80 kd Gamma 2 chain. This generates cells that do not adhere well to connective tissue substrates.

**FGAL:** Non-cleaved variant of Gamma 2 chain. The chain remains intact and is not cleaved at it's BMP-1 site to generate a 155kd chain that restores adhesion.

**Pfu:** A Gamma 2 mutant that encodes cDNA for the constitutively cleaved form of this chain that has been truncated at the proteolytic cleavage site to generate a 105kd chain. These cells do not adhere well to connective tissue substrates.

**Wild type (Gamma 2 WT):** This has a full-length Gamma 2 chain and cells infected with this variant restore their laminin 5 function.

**Delta C115:** This is an empty vector that does not correct laminin 5 due to the absence of Gamma 2 chain and so that cells retain the properties of the mutant cells.

We used the MTT cell proliferation assay to study the effects of SM on JEB cells that were seeded on different BM and ECM components such as Type I Collagen, Type IV Collagen, Fibronectin and Laminin, as well as control plastic plates. Cells were seeded into 24-well plates on these substrates and exposed to 150μM of SM or 1% ethanol on the following day for an exposure time of 7min, rinsed three times with fresh media and incubated for an additional 24 h. Fig. 12 represents results of MTT assays for these JEB cells grown on different ECM or BM substrates. In general, there was an increase in cell viability for the Gamma 2 WT, Delta C115 and FGAL cells, demonstrating that these cells survived the exposure on different substrates to a greater degree when compared to Delta BC and Pfu. However, the degree to which cell viability was altered was dependent on the substrate on which the JEB cells were plated. For example, cells that had restored their laminin 5-mediated adhesive function (FGAL and Gamma 2 WT) showed a minimal loss of viability on Type I and Type IV Collagen and tissue culture plates when compared to JEB cells restored with a gamma 2 chain that did not support adhesion (Delta BC). However, SM-induced loss of cell viability was not as great with Delta BC-restored cells on Fibronectin as had been seen on other substrates. ***These findings demonstrated that restoration of adhesive function mediated by laminin 5 was able to provide decreased cell vulnerability and increased cell survival upon SM exposure.***

In light of these findings, we next performed MTT assays for JEB cells on these substrates at an elevated dose (300μM) of SM and compared this to control exposures of 2% ethanol (Fig. 13). As a control, we compared the SM response of JEB cells to that of normal keratinocytes, as well. JEB cells whose laminin 5-mediated adhesion was restored (FGAL and Gamma 2 WT) showed a similar susceptibility to this dose of SM, as did cells whose adhesion was not restored (Delta BC). Interestingly, all cells grown on Type IV collagen showed a 2 to 3 fold decrease in cell viability even in the presence of 2% ethanol, suggesting that this substrate rendered cells more susceptible to cell death. ***Thus, it appears that restoration of laminin 5 function could provide protection from SM damage only if the SM dose was lower than a threshold amount (150μM) in 2-D culture. Above this dose, even cells with intact laminin 5-mediated adhesion could not withstand SM-induced damage (300μM).***

To further confirm these observations regarding threshold doses of SM, JEB cells were tested on the three substrates that showed the maximum difference in cell viability when treated with SM and ethanol. To accomplish this, JEB cells were exposed to doses of SM (75, 150, 300 μM) and corresponding doses of ethanol controls (0.5, 1, 2 %) for 7 min. Fig. 14 presents results of the MTT assay for the 5 different JEB cell types and human keratinocytes seeded on three different substrates (Type I Collagen, Type IV Collagen, Fibronectin) that were exposed to 75μM of SM or 0.5% ethanol. At lowest SM doses (75μM, 150μM), FGAL cells showed sensitivity to SM damage on Type I Collagen and Fibronectin, but not on Type IV Collagen (Fig. 14). This supported the observation that restoration of laminin 5-mediated adhesion and resistance to M damage were optimal on proteins present in the BM such as Type IV Collagen at low SM doses. This may occur as Type IV Collagen is known to interact with laminin 5 and may provide apoptosis resistance as due to this adhesive association. This also suggested that restoration of laminin 5 function will not reduce the sensitivity to SM damage when cells are grown on a substrate that is not found in BM, such as Type I Collagen. This apoptosis resistance seen for FGAL cells was lost at higher SM doses (300μM, Fig. 16) and was similar to that seen for cells that did not undergo restoration of laminin 5 function (Delta BC) at low SM doses on Type IV Collagen. However, the loss of viability for Delta BC cells was greater than that seen for FGAL cells on Type IV Collage substrates, even at the elevated SM dose (300μM).

A final experiment in monolayer, 2-D culture was performed to compare SM damage between Delta BC and FGAL cells on two substrates (Type I Collagen and Type IV Collagen) that showed the maximum difference in cell viability after SM and ethanol exposure (Fig. 17-20). MTT assays were carried out using 8 different SM doses (75, 150,300, 450, 600, 750, 900, 1200μM) and compared to their corresponding ethanol controls (0.5, 1,2, 3, 4, 6, 8 %). Both FGAL and Delta BC cells showed a threshold, SM dose below which no decrease in cell viability was seen. Surprisingly this threshold was highest (450μM) for Delta BC cells grown on Type IV Collagen plates. In contrast, FGAL cells grown on both Type IV collagen and Type I collagen, as well as Delta BC cells grown on Type I collage showed a small SM-induced decrease in cell viability below 150μM SM. However, as SM dose was increased to 1200μM a gradual dose-dependent decrease in cell viability was seen for both cell types on both Type I and Type IV Collagen substrates.

## PART II: EXPOSURE OF THREE-DIMENSIONAL, HUMAN ORGANOTYPIC CULTURES HARBORING NORMAL HUMAN KERATINOCYTES AND JEB KERATINOCYTES TO DETERMINE THE ROLE OF BASEMENT MEMBRANE ON THE INDUCTION OF SULFUR MUSTARD INJURY

We next generated 3-D, organotypic cultures to identify pathways of SM induced vesication by using engineered human skin that mimics the clinical and histological features of this tissue. Organotypic cultures grown in the absence of pre-existing BM components ("Raft" cultures) were prepared according to our lab protocol. To accomplish this, early passage human dermal fibroblasts were added to neutralized Type I Collagen to a final concentration of $2.5X10^4$ cells per ml. This mixture (3ml) was added to each 35mm well insert of a six-well plate and incubated for 4-6 days in media containing Dulbecco's Modified Eagle's Medium and 10% fetal calf serum, until the collagen matrix showed no further shrinkage. At this time, a total of $5X10^5$ normal human epidermal keratinocytes were seeded directly on the contracted collagen gel. Alternatively, to generate cultures in the presence of BM, cells were seeded onto a de-epidermalized human dermis (AlloDerm) that was layered onto the contracted Type I Collagen gel. Organotypic cultures were maintained submerged in low calcium, epidermal growth media for 2 days , submerged for 2 days in normal calcium epidermal growth media and raised, to the air-liquid interface by feeding tissues from below with cornification media for an additional 2 days. At this point, cultures were exposed to different doses of SM or ethanol that were added to fresh media on day 7 of culture. Tissues were exposed for 7 min based on our previous results with 2-D monolayer cultures (see above). In addition to ethanol treated cultures, untreated cultures were used as controls by not adding ethanol to the media. After exposure, tissues were rinsed three times with fresh media and incubated for an additional 24 h. The following day, tissues were pulsed with 10µm bromodeoxyuridine (BrdU) 6h prior to terminating experiments to allow assay of proliferation in exposed tissues. Tissues were then bisected and one-half was snap-frozen in liquid nitrogen, while the other half of the tissue was formalin-fixed, paraffin-embedded and Hematoxylin and Eosin sections were prepared.

**1. Establishing SM doses that induce tissue damage in 3-D organotypic cultures- morphologic and apoptotic alterations** - To gain an understanding of SM doses needed to induce tissue damage in 3-D tissues, cultures were first treated with 75 and 150µm SM and compared to untreated cultures and those exposed to 1% ethanol. Figures 21 and 22 demonstrate the appearance of collagen Raft cultures grown in the absence of BM that were either unexposed (A), exposed to 75µM (B) or 150µM (C) SM or exposed to 1% ethanol (D). Untreated cultures generated a well-stratified epithelium that adhered to the underlying connective tissue. Tissues treated with 75µM SM demonstrated an intact epithelium that was well-attached to the underlying connective tissue (B). However, these tissues demonstrated a significant degree of altered tissue organization (B) that was similar to that seen for ethanol-exposed cultures (D). In contrast, cultures exposed to 150µM SM demonstrated complete dermal-epidermal separation (C) and an overall thinning of the epithelium. These preliminary findings showed that a 150µm dose of SM for 7 min could mimic the vesicating damaged induced by SM *in vivo.*

To assess the degree of apoptotic cell death in exposed tissues, M30 staining for Rafts exposed to different doses of SM and ethanol was performed from frozen sections. A four-fold increase in apoptotic cells was observed at a dose of 150µM of SM when compared to controls and twice as many apoptotic cells were seen with cultures treated with 75µM of SM (Fig. 23). We next determined if higher SM doses could increase SM-mediated tissue damage in Raft cultures by performing experiments using 150µM and 300µM of SM doses in comparison to 1 and 2% ethanol controls (Fig 24, 25). Low power magnification of H&E stained sections after treatment with 300µM SM showed a significant degree of tissue damage including complete separation of tissue from the BM zone as well as necrosis of keratinocytes and fibroblasts. Less damage was seen in tissues exposed to 150µM SM and there was no separation at the BM interface (Fig.25) To confirm these findings, frozen sections were stained using the M30 antibody. Numbers of apoptotic cells were greatest in tissues exposed to 300μM SM while ethanol-treated tissues showed a very minimal number of apoptotic cells. ***These findings established that doses similar to those found to induce SM damage in 2-D cultures of kerationcytes were also able to induce tissue damage in 3-D organotypic cultures. These tissue alterations included separation of the epithelium at the BM zone in a pattern similar to those found in our in vivo studies (REPORT Year 2).***

***TASK 8 : Dose-time response to establish the role of basement membrane components to SM in 3-D, organotypic cultures***

**<u>2. Establishing the role of basement membrane in human skin response to sulfur mustard</u>** - In light of these findings, we next studied the effects of SM on organotypic cultures grown in the presence and absence of pre-existing BM components. This would allow comparison to MTT assays carried out on different BM components in 2-D cultures described above. Keratinocytes were seeded on AlloDerm, the de-epidermalized, acellular cadaver dermis derived from human skin that forms intact BM at its dermal-epidermal interface. In this way, it would be possible to determine if BM can protect skin-like tissues from SM-induced damage when compared to tissues grown without BM components. To accomplish this, tissues were grown on AlloDerm, collagen Rafts or on polycarbonate membranes coated with either Type I Collagen, Type IV Collage, Fibronectin or control plastic. After 7 days in culture tissues were exposed to SM (150μM) and compared to tissues exposed to 1% ethanol. Fig. 27 represents the low power view of H&E stained tissue sections after SM exposure and Fig. 28 represents the higher magnification view. SM at a dose of 150μM induced separation at the BM zone when both collagen Raft (Fig. 27 B, Fig. 28 C) and plastic, non-coated inserts (Fig. 27 D, Fig. 28 B) were compared to the ethanol control (Fig. 27 D, E, Fig. 28 B, D). In contrast, tissues grown on AlloDerm showed that the BM interface was intact (Fig. 27 C, Fig. 28 E) as seen in ethanol-exposed controls (Fig. 27 F, Fib. 28F). Similarly, SM induced separation at the BM zone for tissues grown on different substrates (Type I Collagen, Type IV Collagen, Fibronectin) when compared to ethanol controls (Fig. 29 and Fig. 30).

To confirm these findings a dose of 150μM SM was used and compared to 1% ethanol and untreated controls to compare the effect of SM on organotypic cultures on which keratinocytes were grown on Rafts and AlloDerm (Fig. 31 and 32). Tissues treated with 150μM SM demonstrated intact tissues (Fig. 31 B) with minimal numbers of damaged, eosinophilic cells in the supra-basal layer (Fig. 32 B arrows) while untreated tissues showed a well-stratified epithelium that was similar to ethanol controls. In contrast, SM-treated Rafts showed a significantly higher number of damaged keratinocytes that displayed nuclear condensation and eosinophilic cytoplasm proving that SM could induce more severe damage to cells grown on Rafts than those grown on AlloDerm. To confirm these findings, we performed immunofluorescent staining using the M30 antibody to assess numbers of apoptotic cells in exposed tissues (Fig. 33 & 34). Rafts treated with SM showed a 10-fold increase in number of apoptotic cells when compared to AlloDerm (Fig. 34), which displayed number of apoptotic cells that were similar to non-treated and ethanol controls. To determine if the induction of apoptosis demonstrated a dose-dependency, we performed experiments on AlloDerm using two different doses of SM along and compared them to controls. AlloDerms exposed to doses of 75μM and 150μM of SM were similar to untreated and ethanol controls in both tissue morphology (Fig. 35, 36) and upon M30 staining and numbers of apoptotic cells after M30 staining when compared to controls (Fig. 37).

In light of these findings, we next determined the minimal SM dose required for the induction of tissue damage in AlloDerm cultures. We selected 5 different SM doses (75, 150, 300, 600, 1200 μM) and compared these to ethanol controls (0.5, 1, 2, 4, 8 %) (Fig. 38 and 39). We found that the epithelium remained intact at SM doses of 75 and 150μM as induction of SM-mediated damage started at a dose of 300μM. At elevated doses of 600 and 1200μM SM tissue alterations were more prominent and were characterized by separation of the BM zone. Ethanol treated cultures showed little tissue damage even at elevated levels of ethanol exposure. M30 staining of these cultures showed a gradual increase in the number of apoptotic cells with increasing doses of SM from 75 to 300μM SM (Fig. 41). An SM dose of 75μM showed 3.8% of apoptotic cells compared to 57.2% observed for 1200μM while the maximum percentage of apoptotic cells observed for the highest dose of ethanol (8%) was only 3.6%. This gradual increase in numbers of apoptotic cells showed that AlloDerm tissues were susceptible to SM, but only above a threshold level of SM (600μM). ***These findings showed that only tissues with an intact BM could resist SM-induced damage, thus demonstrating that BM structure was protective upon SM exposure. This protective effect was only seen at doses of SM below 600 uM.***

***TASK 5: Dose-time response to establish the response of JEB tissue constructs which were reverted to a normal phenotype by retroviral gene transfer to SM***
***TASK 6: Assay the response of JEB and normal organotypic cultures to high, vesicating doses of SM***
***TASK 7: Assay the response of JEB keratinocytes expressing mutated forms of the gamma- chain of laminin 5***

***3. Establishing the role of laminin 5-mediated adhesion in the response of 3-D tissues to sulfur mustard – the response of JEB mutants to sulfur mustard in 3-D tissues -*** Since it appeared that BM could protect tissues exposed to SM, we performed a final experiment to determine if JEB cells grown on AlloDerm demonstrated a differential susceptibility to 150μM SM (Fig. 42 and 43). All JEB cells showed a degree of epithelial separation at the BM zone when compared to ethanol-exposed control cultures. Normal cells grown on AlloDerm showed protection from SM damage but AlloDerm did not protect JEB cells, (FGAL, Delta BC, Pfu, Delta C115 and Gamma2wt) against the SM induced damage. This was confirmed by M30 staining of these tissues where we observed the induction of apoptosis due to SM compared to ethanol controls (Fig. 45). Paradoxically, FGAL and Gamma 2 WT cells showed the greater number of apoptotic cells among all different JEB cell types. ***This suggests that restoration at laminin 5-mediated adhesion did not protect tissues from SM-induced damage when tissues were exposed to 150μM SM and that restoration of laminin 5 alone was not sufficient to provide protection from the effects of SM.***

## KEY RESEARCH ACCOMPLISHMENTS:

1 - We established dose/time responses of normal keratinocytes following exposure to SM and ethanol vehicle in tissue culture media (150uM) that induced biologically-meaningful changes in cell apoptosis and cell viability in 2-D cultures.
2 - We established dose/time responses of JEB keratinocytes following exposure to SM and ethanol vehicle in tissue culture media (150uM) that induced biologically-meaningful changes in cell apoptosis and cell viability in 2-D cultures.
3 - We have that established cells grown on the BM component Type IV Collagen demonstrated the greatest sensitivity of cells to SM-induced damage in 2-D cultures. These findings demonstrated that

individual ECM or BM components were not able to provide protection from SM damage in 2-D cultures.

4 – We have found that restoration of laminin 5 function could provide protection from SM damage only if the SM dose was lower than a threshold amount (150 μM in 2-D) culture. Above this dose, even cells with intact laminin 5-mediated adhesion could not withstand SM-induced damage (300μM).

5 – We determined that doses similar to those found to induce SM damage in 2-D cultures of kerationcytes were also able to induce tissue damage in 3-D organotypic cultures. These tissue alterations included separation of the epithelium at the BM zone in a pattern similar to those found in our in vivo studies (REPORT Year 2).

6 – We have found that showed that only tissues with an intact BM could resist SM-induced damage, thus demonstrating that BM structure was protective upon SM exposure. This protective effect was only seen at doses of SM below 600 uM.

7 - We found that restoration at laminin 5-mediated adhesion did not protect tissues from SM-induced damage when tissues were exposed to 150μM SM and that restoration of laminin 5 alone was not sufficient to provide protection from the effects of SM..

# REPORTABLE OUTCOMES

## PAPERS

1. Andriani, F., Margulis, A., Lin, N., Griffey, S., and Garlick, J.A. Analysis of Microenvironmental Factors Contributing to Basement Membrane Assembly and Normalized Epidermal Phenotype, J. INVEST. DERMATOL. 120:923-931, 2003.

2. Greenberg, S., Prabhu, P., Garfield, J., Hamilton, T., Petrali, J., and Garlick, J.A. Characterization of the Initial Response of Bioengineered Human Skin to Sulfur Mustard: The Role of Basement Membrane (U.S. Army Medical Defense Review Bioscience, 2004).

## ABSTRACTS PRESENTED

1. Prabhu, P., Greenberg, S., Lin, N., Garfield, J., Hamilton, T., Petrali, J., and Garlick, J.A. Characterization of the Initial Response of Engineered Human Skin to Sulfur Mustard (Society for Investigative Dermatology, 2004)

2. Kamath, P., Greenberg, S., Petrali, J., Hamilton, T., Garfield, J., Pommeret, O., Meneguzzi, G., and Garlick, J.A. Characterization of the Initial Response of Bioengineered Human Skin to Sulfur Mustard: The Role of Basement Membrane (U.S. Army Medical Defense Review Bioscience, 2004)

# CONCLUSIONS

A major goal of our research studies was to determine the initiating site of SM induced damage that leads to vesicating injury in human skin. YEAR 1 of our research allowed us to generate optimized *in vitro* and *in vivo* human tissue models harboring basement membrane that have made many of the discoveries in this report possible. YEAR 2 of this research focused on characterization of the *in vivo* response of bioengineered human skin to prevesicating and vesicating doses of SM.

During this year of our research, we received approval for the use of an in-house facility for SM exposures. This laboratory was constructed during YEARS 1 and 2 of our research and final approval for its use was obtained one year ago. This facility greatly facilitated the progress of our work as it allowed all studies with SM to be performed in our laboratory. YEAR 3 of our research has allowed us to establish that intact basement membrane significantly reduces the vulnerability of 3D, human skin-like tissues to vesicating injury. The finding of an increased susceptibility of human skin-like tissues without structured basement membrane to SM-induced vesication indicates that this structure is a critical site for the initiation of SM injury in human skin. Studies in 2D cultures demonstrated that the presence of proteins found in the cutaneous basement membrane zone were not able to reduce the sensitivity to SM damage. In fact, quite the opposite was true, as we determined that cell viability (MTT assay) was lowered and apoptosis was increased (M30 assay) when cells were grown in 2D culture on the basement membrane component Type IV collagen and exposed to 150μM of SM. Since it is known that human epidermal keratinocytes have elevated growth on Type IV Collagen, it is possible that this increased proliferation was associated with the elevation of cell damage. Similarly, even in 3D cultures, the presence of basement membrane proteins that were not organized into structured basement membrane were not sufficient to protect keratinocytes from SM damage. We concluded that it was critical to have well-structured basement membrane in 3D (AlloDerm) cultures in order to prevent SM damage leading to vesication. To further establish the role of the basement membrane as a mediator of SM-induced vulnerability, we focused on the role of laminin 5. By using JEB cells restored with a variety of $\gamma 2$ chain retroviral constructs, we concluded that restoration of adhesive function mediated by laminin 5 could decrease susceptibility to SM injury. These findings directly implicate laminin 5 and its role in structured basement membrane that mediate adhesive interaction that can prevent SM-induced vesication and can directly modulate SM injury in human skin.

# Chart 1:Colony forming efficiency test for diff. Doses of Ethanol.

**Plate 1**

| Obj.# | Area | Diameter (mean) |
|---|---|---|
| 1 | 303 | 19.01095 |
| 3 | 431 | 22.4343 |
| 4 | 297 | 18.57384 |
| 5 | 655 | 27.72862 |
| 6 | 719 | 29.17088 |
| 7 | 981 | 34.67185 |
| 8 | 966 | 32.08782 |
| 9 | 100 | 10.25018 |
| 10 | 218 | 15.93938 |
| 11 | 248 | 17.03653 |
| 12 | 421 | 22.1994 |
| 14 | 341 | 19.97868 |
| 15 | 108 | 11.67423 |
| 16 | 575 | 25.96036 |
| 17 | 257 | 17.30348 |
| 19 | 466 | 23.09092 |
| 22 | 340 | 20.34564 |
| 23 | 252 | 17.48212 |
| 24 | 749 | 31.67792 |
| 26 | 295 | 18.8583 |
| 27 | 417 | 21.10617 |
| 28 | 500 | 24.01897 |
| 29 | 908 | 33.62228 |
| 33 | 190 | 15.03517 |
| 34 | 131 | 14.35246 |
| 39 | 379 | 21.48697 |
| 41 | 599 | 26.66582 |
| 43 | 495 | 24.06542 |
| 46 | 338 | 19.79765 |
| 47 | 204 | 15.55703 |
| 50 | 153 | 12.24627 |
| 53 | 196 | 14.63457 |
| 56 | 799 | 31.08588 |
| 57 | 486 | 23.7671 |
| 61 | 756 | 29.99797 |
| 64 | 370 | 20.51874 |
| 65 | 1285 | 40.55771 |
| 69 | 270 | 17.70592 |
| 73 | 147 | 12.24352 |
| 78 | 196 | 14.86915 |
| 79 | 254 | 16.86913 |
| 82 | 604 | 26.59736 |
| 85 | 531 | 25.03112 |
| 86 | 658 | 27.99984 |
| 90 | 281 | 17.53221 |
| 94 | 232 | 16.66273 |
| 95 | 524 | 24.8019 |
| 96 | 576 | 25.94113 |
| 102 | 298 | 18.77829 |
| 108 | 229 | 16.27969 |
| Stats | Area | Diameter (mean) |
| Min | 100 | 10.25018 |
| (Obj.#) | 9 | 9 |
| Max | 1285 | 40.55771 |
| (Obj.#) | 65 | 65 |
| Range | 1185 | 30.30754 |
| Mean | 434.56 | 21.70607 |
| Std.Dev | 256.4732 | 6.597242 |
| Sum | 21728 | 1085.304 |

**Plate 2**

| Obj.# | Area | Diameter (mean) |
|---|---|---|
| 1 | 723 | 29.23695 |
| 2 | 981 | 35.07506 |
| 3 | 274 | 17.98097 |
| 4 | 497 | 23.99 |
| 5 | 271 | 17.63542 |
| 6 | 1158 | 38.17083 |
| 7 | 201 | 15.18621 |
| 8 | 1484 | 42.61647 |
| 10 | 784 | 31.45005 |
| 12 | 753 | 30.25857 |
| 13 | 1594 | 44.31307 |
| 14 | 327 | 19.86307 |
| 15 | 705 | 28.99963 |
| 16 | 213 | 14.60063 |
| 17 | 560 | 25.71567 |
| 18 | 885 | 32.77757 |
| 19 | 302 | 18.4119 |
| 20 | 1359 | 40.73583 |
| 21 | 731 | 29.84407 |
| 23 | 314 | 19.1414 |
| 24 | 567 | 25.63724 |
| 25 | 1278 | 39.90689 |
| 27 | 230 | 16.0985 |
| 28 | 847 | 33.12015 |
| 29 | 256 | 17.58948 |
| 30 | 1730 | 45.71595 |
| 32 | 196 | 15.62357 |
| 33 | 813 | 31.70016 |
| 34 | 982 | 35.82237 |
| 36 | 826 | 33.93631 |
| 38 | 246 | 16.62804 |
| 40 | 908 | 32.95882 |
| 41 | 1714 | 45.46995 |
| 42 | 985 | 34.64849 |
| 43 | 915 | 33.03319 |
| 44 | 1123 | 36.98126 |
| 45 | 343 | 20.56274 |
| 47 | 736 | 29.52664 |
| 48 | 1019 | 35.03419 |
| 49 | 487 | 23.96436 |
| 50 | 634 | 27.07093 |
| 51 | 1322 | 41.54865 |
| 52 | 1726 | 45.68951 |
| 53 | 441 | 22.72913 |
| Stats | Area | Diameter (mean) |
| Min | 196 | 14.60063 |
| (Obj.#) | 32 | 16 |
| Max | 1730 | 45.71595 |
| (Obj.#) | 30 | 30 |
| Range | 1534 | 31.11533 |
| Mean | 782.7273 | 29.47727 |
| Std.Dev | 447.8669 | 9.309393 |
| Sum | 34440 | 1297 |
| Samples | 44 | 44 |

**Plate 3**

| Obj.# | Area | Diameter (i |
|---|---|---|
| 1 | 368 | 21.00228 |
| 2 | 1452 | 42.55793 |
| 5 | 1369 | 40.98646 |
| 6 | 635 | 27.42119 |
| 7 | 190 | 14.83235 |
| 12 | 788 | 30.52805 |
| 13 | 620 | 26.82806 |
| 14 | 561 | 25.79769 |
| 15 | 1358 | 41.23758 |
| 16 | 354 | 20.27196 |
| 17 | 1117 | 36.77989 |
| 18 | 223 | 16.18283 |
| 19 | 848 | 31.66589 |
| 20 | 557 | 25.61567 |
| 21 | 397 | 21.52352 |
| 22 | 564 | 25.59877 |
| 23 | 1003 | 36.18402 |
| 25 | 524 | 24.80816 |
| 26 | 414 | 21.91118 |
| 27 | 800 | 30.98713 |
| 28 | 838 | 31.67329 |
| 29 | 263 | 17.29511 |
| 30 | 1027 | 36.47375 |
| 31 | 735 | 29.40827 |
| 32 | 1570 | 43.80937 |
| 33 | 1128 | 37.05792 |
| 35 | 395 | 21.51804 |
| 36 | 436 | 22.78733 |
| 37 | 502 | 24.43275 |
| 39 | 364 | 20.66486 |
| 40 | 385 | 21.19656 |
| 41 | 336 | 20.19264 |
| 42 | 480 | 23.92561 |
| 43 | 302 | 18.93908 |
| 44 | 779 | 31.15737 |
| 45 | 1301 | 39.77202 |
| 47 | 929 | 33.56456 |
| 48 | 246 | 16.91179 |
| 49 | 326 | 19.43613 |
| 52 | 774 | 30.40629 |
| 53 | 935 | 33.47803 |
| 54 | 285 | 18.11605 |
| 57 | 238 | 16.54171 |
| 58 | 420 | 21.98574 |
| 59 | 1082 | 37.68928 |
| Stats | Area | Diameter (i |
| Min | 190 | 14.83235 |
| (Obj.#) | 7 | 7 |
| Max | 1570 | 43.80937 |
| (Obj.#) | 32 | 32 |
| Range | 1380 | 28.97702 |
| Mean | 671.5111 | 27.35898 |
| Std.Dev | 369.8065 | 8.007271 |
| Sum | 30218 | 1231.154 |
| Samples | 45 | 45 |

**Plate 4**

| Obj.# | Area | Diameter (mean) |
|---|---|---|
| 1 | 693 | 28.66456 |
| 2 | 606 | 26.8391 |
| 3 | 633 | 27.27014 |
| 4 | 898 | 32.7391 |
| 5 | 685 | 28.54366 |
| 6 | 299 | 18.61875 |
| 7 | 922 | 34.17847 |
| 8 | 666 | 28.09636 |
| 9 | 1131 | 38.86394 |
| 10 | 230 | 16.5682 |
| 11 | 329 | 19.83361 |
| 12 | 651 | 27.7603 |
| 13 | 773 | 31.61734 |
| 14 | 778 | 30.46585 |
| 15 | 245 | 16.73562 |
| 16 | 848 | 32.20823 |
| 17 | 447 | 23.38809 |
| 18 | 1079 | 36.99848 |
| 19 | 917 | 34.32692 |
| 20 | 570 | 26.36699 |
| 21 | 888 | 34.27879 |
| 22 | 429 | 22.59546 |
| 23 | 1645 | 44.49541 |
| 24 | 266 | 18.10159 |
| 26 | 434 | 22.32742 |
| 30 | 316 | 19.36658 |
| 31 | 483 | 23.76301 |
| 34 | 641 | 27.40071 |
| 35 | 693 | 28.70903 |
| 36 | 497 | 23.99812 |
| 39 | 923 | 33.45653 |
| 40 | 515 | 24.64205 |
| 41 | 906 | 33.00581 |
| 42 | 506 | 24.66411 |
| 43 | 529 | 24.95889 |
| 45 | 397 | 21.63952 |
| 46 | 304 | 19.0132 |
| 47 | 541 | 25.22305 |
| 48 | 823 | 31.3452 |
| 49 | 802 | 30.86987 |
| 51 | 727 | 29.21968 |
| 52 | 765 | 29.94201 |
| Stats | Area | Diameter (mean) |
| Min | 230 | 16.5682 |
| (Obj.#) | 10 | 10 |
| Max | 1645 | 44.49541 |
| (Obj.#) | 23 | 23 |
| Range | 1415 | 27.9272 |
| Mean | 653.0952 | 27.45476 |
| Std.Dev | 277.2394 | 6.139822 |
| Sum | 27430 | 1153.1 |
| Samples | 42 | 42 |

**Plate 5**

| Obj.# | Area | Diameter (mean) |
|---|---|---|
| 2 | 333 | 19.88689 |
| 3 | 457 | 23.60689 |
| 4 | 572 | 25.84705 |
| 10 | 705 | 28.69458 |
| 13 | 1663 | 45.83457 |
| 14 | 587 | 26.44698 |
| 15 | 1606 | 44.38457 |
| 16 | 260 | 18.25182 |
| 17 | 1606 | 44.10838 |
| 18 | 420 | 22.20976 |
| 19 | 1511 | 42.74728 |
| 20 | 1141 | 37.76366 |
| 21 | 996 | 34.7277 |
| 22 | 378 | 21.82349 |
| 24 | 278 | 18.55956 |
| 28 | 330 | 19.70147 |
| 29 | 385 | 20.99992 |
| 30 | 256 | 17.35729 |
| 31 | 653 | 27.54719 |
| 33 | 1090 | 36.01027 |
| 36 | 639 | 27.4251 |
| 37 | 1852 | 47.57377 |
| 38 | 472 | 23.53242 |
| 39 | 1373 | 40.96551 |
| 40 | 551 | 25.44096 |
| 41 | 574 | 26.1926 |
| 42 | 1032 | 35.2921 |
| 44 | 496 | 24.02659 |
| 45 | 2182 | 54.34765 |
| 46 | 349 | 20.1659 |
| 48 | 680 | 28.37125 |
| 51 | 287 | 18.14533 |
| 53 | 551 | 25.46806 |
| 55 | 371 | 21.15667 |
| 56 | 417 | 21.87907 |
| 58 | 498 | 23.98555 |
| 63 | 805 | 31.01447 |
| 64 | 332 | 19.79741 |
| Stats | Area | Diameter (mean) |
| Min | 256 | 17.35729 |
| (Obj.#) | 30 | 30 |
| Max | 2182 | 54.34765 |
| (Obj.#) | 45 | 45 |
| Range | 1926 | 36.99036 |
| Mean | 754.9474 | 28.71815 |
| Std.Dev | 504.5143 | 9.617395 |
| Sum | 28688 | 1091.29 |
| Samples | 38 | 38 |

**Plate 6**

| Obj.# | Area | Diameter (mean) |
|---|---|---|
| 1 | 685 | 28.4904 |
| 2 | 369 | 20.75996 |
| 3 | 1981 | 49.6653 |
| 4 | 240 | 16.68826 |
| 5 | 252 | 17.42439 |
| 6 | 616 | 27.25112 |
| 7 | 356 | 20.58161 |
| 8 | 385 | 20.79862 |
| 9 | 949 | 35.24616 |
| 10 | 351 | 20.38016 |
| 11 | 333 | 19.79145 |
| 12 | 603 | 26.66379 |
| 13 | 1556 | 43.41113 |
| 14 | 679 | 28.46158 |
| 20 | 957 | 31.19653 |
| 24 | 203 | 15.02552 |
| 25 | 657 | 28.20578 |
| 26 | 617 | 26.88225 |
| 27 | 965 | 34.37101 |
| 29 | 1465 | 42.81949 |
| 30 | 257 | 17.93176 |
| 32 | 1405 | 40.98995 |
| 33 | 2525 | 54.89707 |
| 34 | 308 | 19.23189 |
| 35 | 234 | 16.08917 |
| 36 | 345 | 19.85205 |
| 37 | 729 | 29.34691 |
| 38 | 475 | 23.76461 |
| 39 | 341 | 19.80422 |
| 40 | 885 | 33.29909 |
| 41 | 361 | 20.52434 |
| 42 | 410 | 21.87765 |
| 43 | 228 | 16.20058 |
| 44 | 1404 | 41.41729 |
| 45 | 301 | 19.06744 |
| 47 | 926 | 33.60464 |
| 48 | 362 | 20.48055 |
| 49 | 435 | 22.32147 |
| 50 | 232 | 16.60924 |
| 51 | 323 | 19.33572 |
| Stats | Area | Diameter (mean) |
| Min | 203 | 15.02552 |
| (Obj.#) | 24 | 24 |
| Max | 2525 | 54.89707 |
| (Obj.#) | 33 | 33 |
| Range | 2322 | 39.87155 |
| Mean | 667.125 | 26.519 |
| Std.Dev | 519.721 | 9.810958 |
| Sum | 26685 | 1060.76 |
| Samples | 40 | 40 |

# Chart 1:Colony forming efficiency test for diff. Doses of Ethanol.

**Plate 7**

| Obj.# | Area | Diameter (mean) |
|---|---|---|
| 1 | 311 | 18.84619 |
| 2 | 318 | 19.13212 |
| 3 | 1861 | 47.4571 |
| 4 | 319 | 19.42469 |
| 6 | 338 | 20.03454 |
| 7 | 2393 | 53.89959 |
| 8 | 319 | 19.38202 |
| 9 | 2921 | 60.42813 |
| 10 | 1095 | 36.68781 |
| 12 | 668 | 28.04055 |
| 13 | 377 | 20.92064 |
| 15 | 2657 | 56.46736 |
| 18 | 314 | 19.39491 |
| 21 | 1229 | 38.13656 |
| 22 | 1235 | 38.62394 |
| 23 | 470 | 23.73361 |
| 24 | 1179 | 39.52578 |
| 25 | 564 | 25.93602 |
| 27 | 637 | 27.13687 |
| 29 | 499 | 24.28621 |
| 30 | 2715 | 57.31591 |
| 31 | 521 | 24.75276 |
| 32 | 808 | 30.94194 |
| 35 | 320 | 19.49257 |
| 36 | 243 | 16.84583 |
| 38 | 591 | 27.60254 |
| 39 | 350 | 20.67114 |
| 41 | 404 | 21.85555 |
| 43 | 410 | 21.99871 |
| 44 | 278 | 17.76215 |
| 45 | 620 | 27.06714 |
| 46 | 3746 | 68.06725 |
| 47 | 981 | 34.38712 |
| 48 | 757 | 29.96356 |
| 49 | 2014 | 49.61019 |
| 50 | 1735 | 46.23339 |
| Stats | Area | Diameter (mean) |
| Min | 243 | 16.84583 |
| (Obj.#) | 36 | 36 |
| Max | 3746 | 68.06725 |
| (Obj.#) | 46 | 46 |
| Range | 3503 | 51.22141 |
| Mean | 1005.472 | 32.00173 |
| Std.Dev | 893.8641 | 13.99589 |
| Sum | 36197 | 1152.062 |
| Samples | 36 | 36 |

**Plate 8**

| Obj.# | Area | Diameter (ı |
|---|---|---|
| 1 | 981 | 34.25568 |
| 2 | 664 | 27.40764 |
| 3 | 627 | 29.62269 |
| 4 | 867 | 32.62684 |
| 5 | 1857 | 47.70511 |
| 6 | 724 | 29.20603 |
| 7 | 1074 | 37.28857 |
| 8 | 1434 | 41.99668 |
| 9 | 526 | 24.80192 |
| 10 | 671 | 28.0817 |
| 11 | 956 | 34.29499 |
| 12 | 977 | 34.44915 |
| 13 | 564 | 25.91216 |
| 14 | 208 | 15.60507 |
| 15 | 1547 | 44.14896 |
| 16 | 452 | 23.18513 |
| 17 | 378 | 20.97375 |
| 19 | 1376 | 41.49363 |
| 20 | 612 | 26.79121 |
| 21 | 284 | 18.60869 |
| 22 | 2120 | 50.5307 |
| 23 | 874 | 32.32846 |
| 24 | 2446 | 54.7328 |
| 25 | 790 | 30.42181 |
| 26 | 1243 | 39.00727 |
| 27 | 1324 | 41.81233 |
| 29 | 489 | 23.73582 |
| 30 | 805 | 30.57047 |
| 32 | 266 | 18.03897 |
| 34 | 398 | 21.72645 |
| 37 | 904 | 34.10687 |
| 38 | 1326 | 40.3833 |
| 39 | 891 | 33.26693 |
| Stats | Area | Diameter (ı |
| Min | 208 | 15.60507 |
| (Obj.#) | 14 | 14 |
| Max | 2446 | 54.7328 |
| (Obj.#) | 24 | 24 |
| Range | 2238 | 39.12773 |
| Mean | 928.9394 | 32.39751 |
| Std.Dev | 520.7398 | 9.362057 |
| Sum | 30655 | 1069.118 |
| Samples | 33 | 33 |

# Fig. 1: Colony forming efficiency test for diff. Doses of Ethanol.

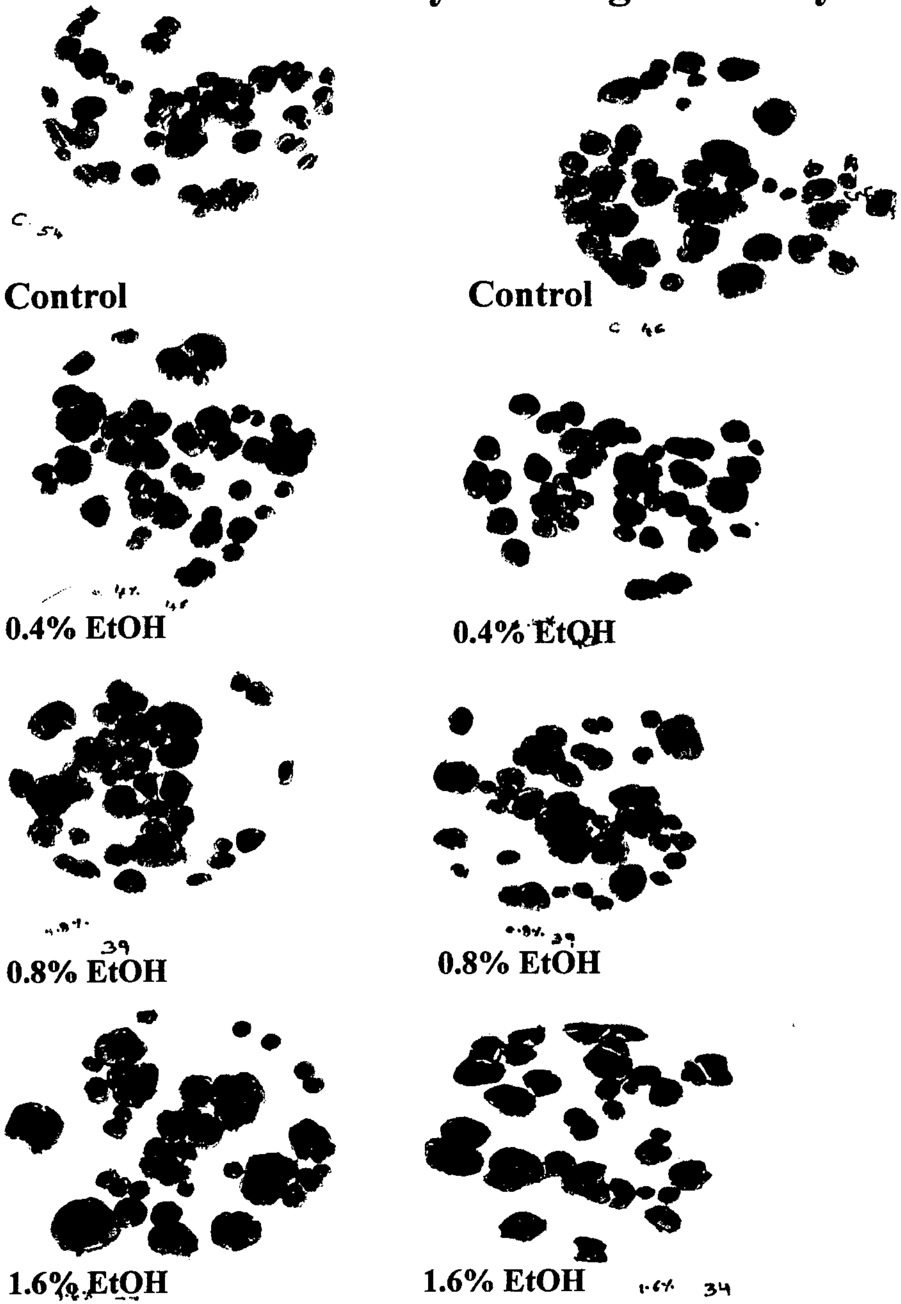



**Fig. 1: Colonies shown by the Keratinocytes after the exposure to different doses of ethanol (0.4%, 0.8%, 1.6%) along with untreated control**

**Fig.2: Graphical representation of colony forming efficiency of keratinocytes exposed to diff.doses of ethanol.**

**Table:**

| Chemicals | Ave. no. of colonies. |
|---|---|
| Control | 50 |
| 0.4%EtOH | 45 |
| 0.8%EtOH | 39 |
| 1.6%EtOH | 35 |

**Graph:**

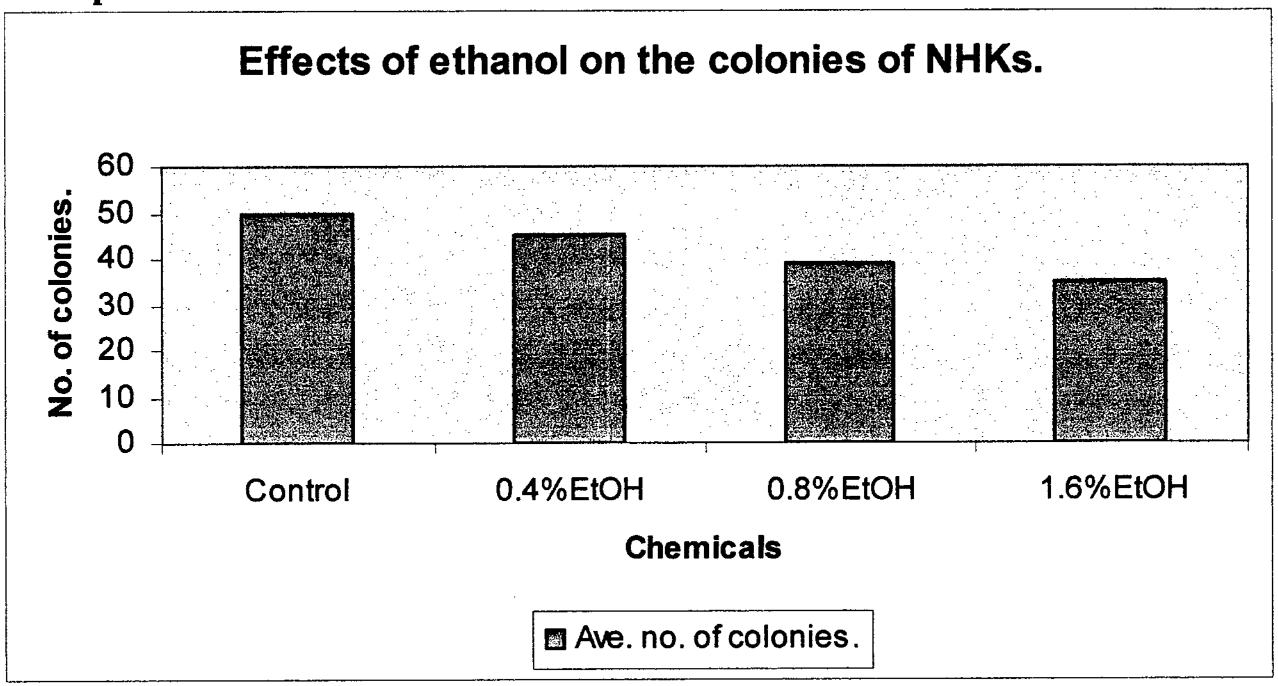
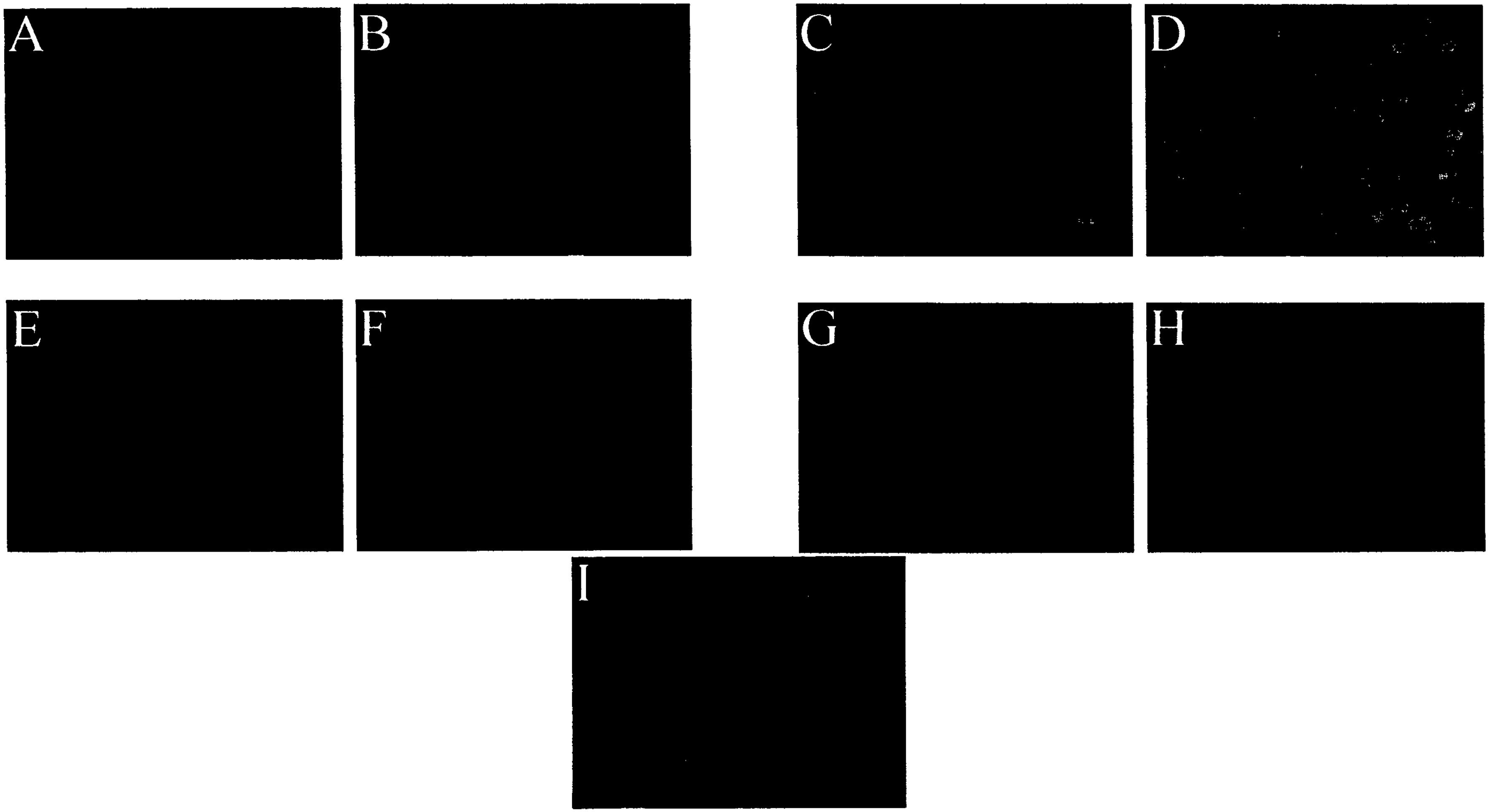


**Fig. 3: Immunofluorescent staining for Apoptotic cells induced by diff. Doses of SM, Ethanol and control.**

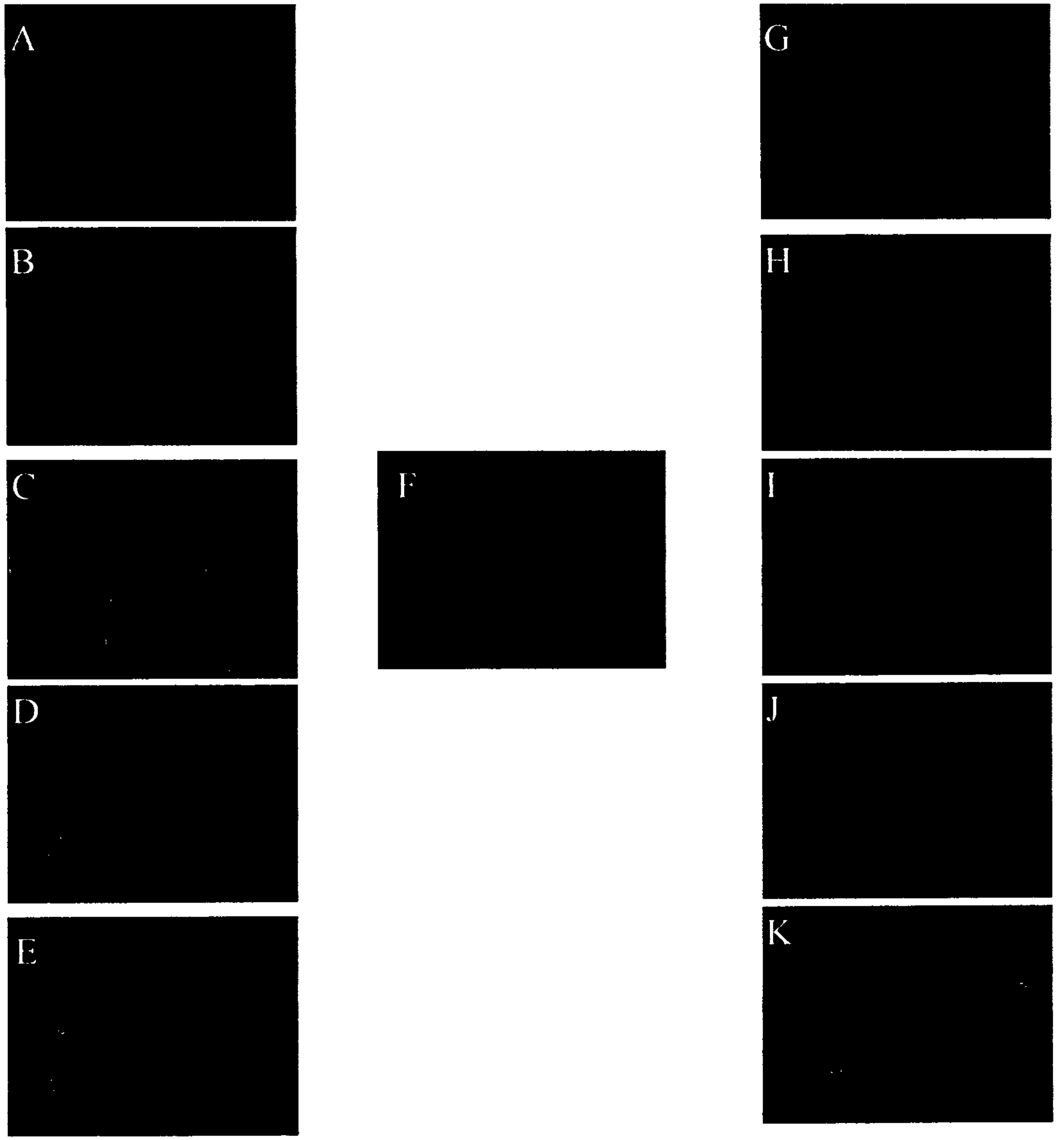



**A-D** -Varying doses of SM (37.5, 75, 150, 300*u*m)

**E-H** – Varying doses of concurrent Ethanol controls (0.25, 0.5, 1, 2%)

**I** – Untreated control.

**Fig. 4: M30 Cytodeath staining for Keratinocytes exposed SM and Ethanol at diff. Sample intervals.**




**A-E**– 1, 3, 7, 14, 28 min. of 150um of SM; **G-K** – 1, 3, 7, 14, 28 min of 1% Ethanol;

**F**– Untreated control.

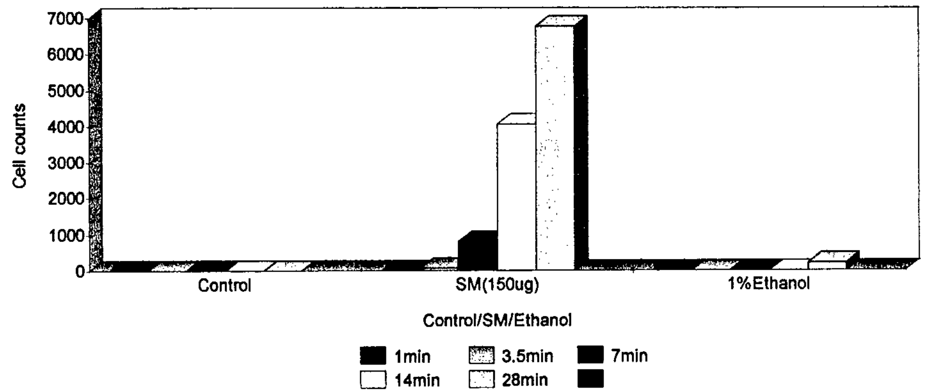
# Fig. 5: Apoptotic cell counts (NHK)

## Table:

| Chemicals | 1min. | 3.5min. | 7min. | 14min. | 28min. |
|---|---|---|---|---|---|
| Control | 4 | 4 | 4 | 4 | 4 |
| SM (150ug) | 18 | 69 | 795 | 4015 | 6783 |
| 1%EtOH | 6 | 13 | 15 | 18 | 221 |

**Apoptotic Cell Counts for SM**

M30 stained NHK colonies on coverslip




**Apoptotic Cell counts**

Control, SM, Ethanol (Comparision)

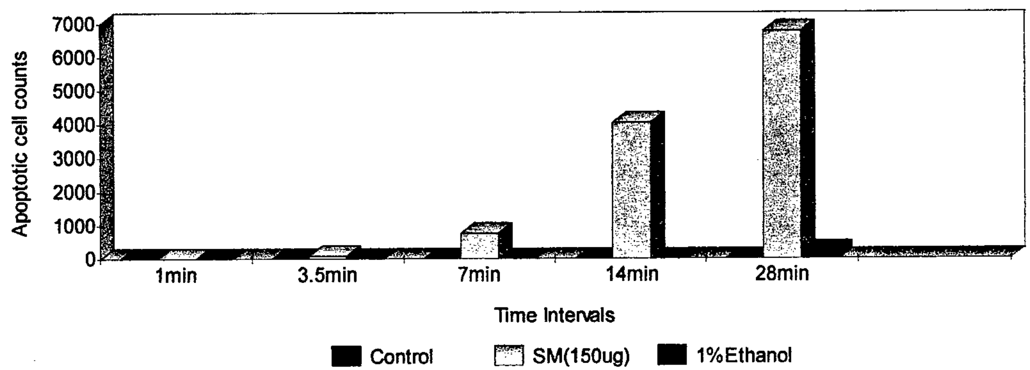
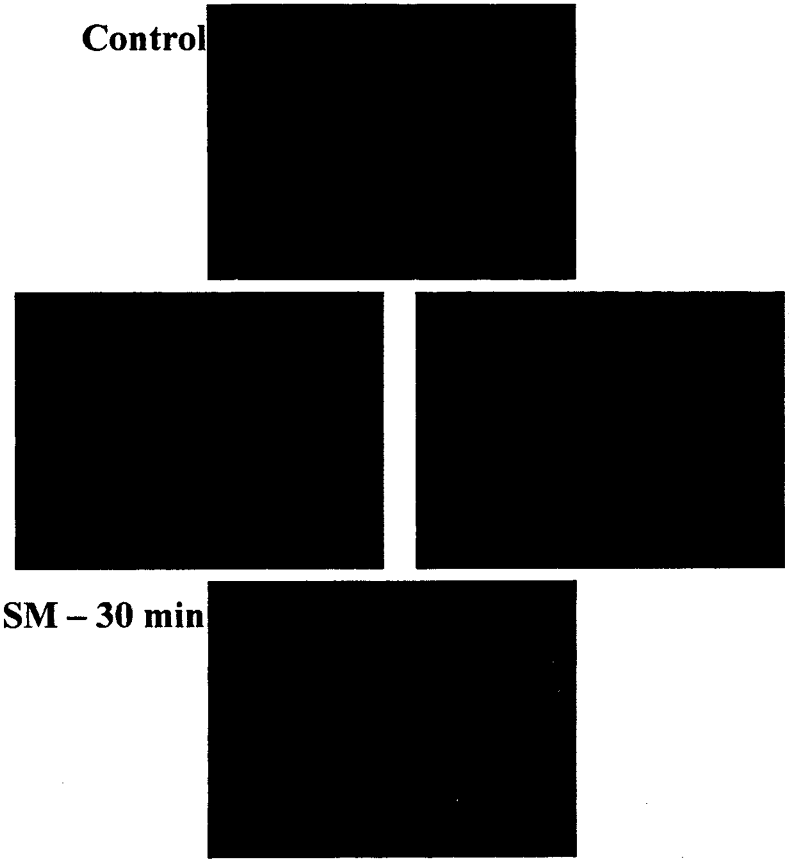


**Fig. 6: M30 Cytodeath staining for 150$u$m SM and 1% Ethanol at two different intervals.**



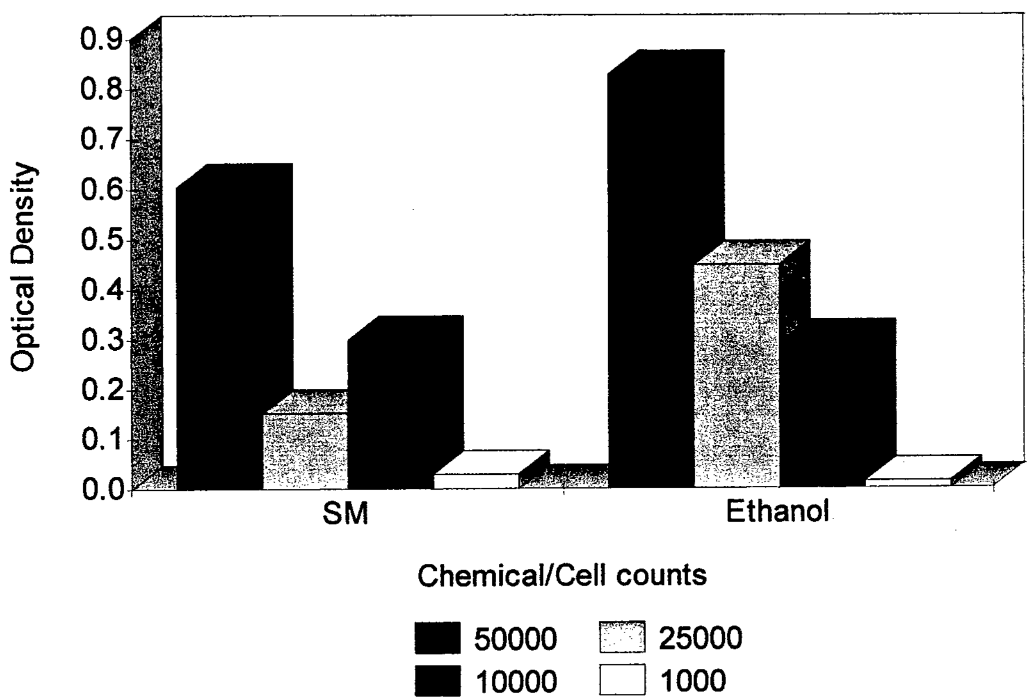
# Fig.7: MTT Assay for NHK's on Plastic plates at diff. Cell density.

**Plastic**

| **Chemica** | **50000** | **25000** | **10000** | **1000** |
|---|---|---|---|---|
| **SM** | **0.605** | 0.153 | 0.302 | 0.029 |
| **Ethanol** | **0.828** | 0.449 | 0.289 | 0.013 |

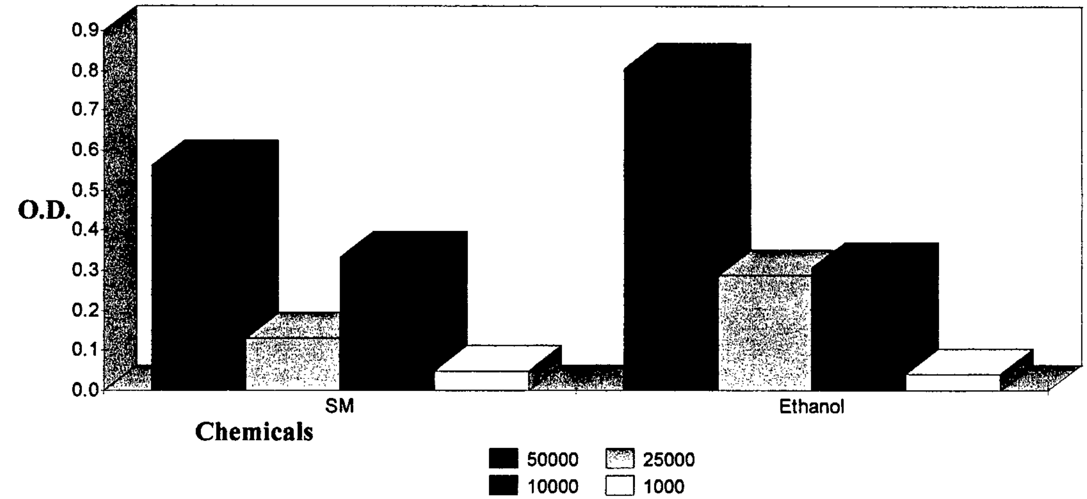
**MTT Assay for NHKs on Plastic plate (24**

SM (150μm for 7min); 1% Ethanol for



# Fig. 8: MTT Assay for NHK's at varied Cell density on Type IVCollagen plates.

| Chemica | 50000 | 25000 | 10000 | 1000 |
|---|---|---|---|---|
| SM | **0.564** | 0.133 | 0.332 | 0.047 |
| Ethanol | **0.807** | 0.287 | 0.307 | 0.0413 |



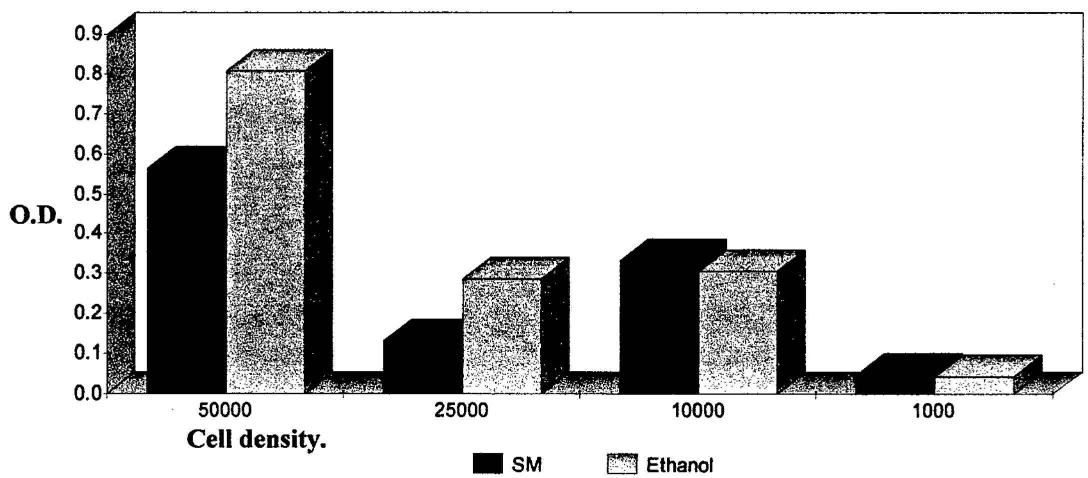
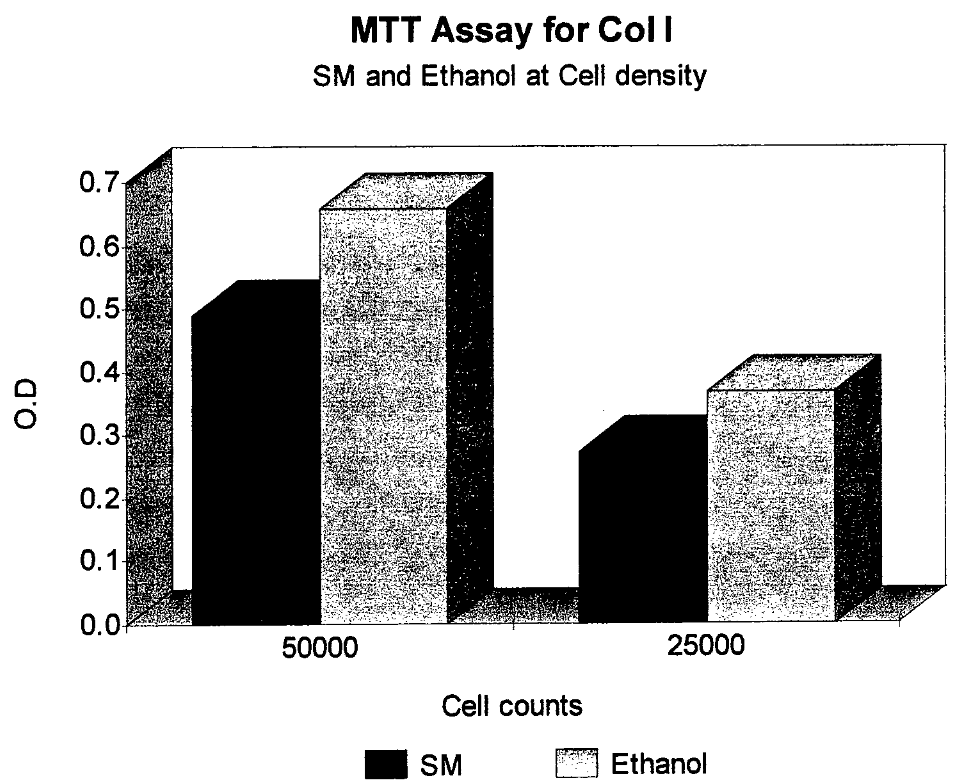



Fig. 9: MTT Assay at two different cell densities of NHK's on Col I

| Chemica | 50000 | 25000 |
|---|---|---|
| SM | 0.491 | 0.272 |
| Ethanol | 0.657 | 0.369 |

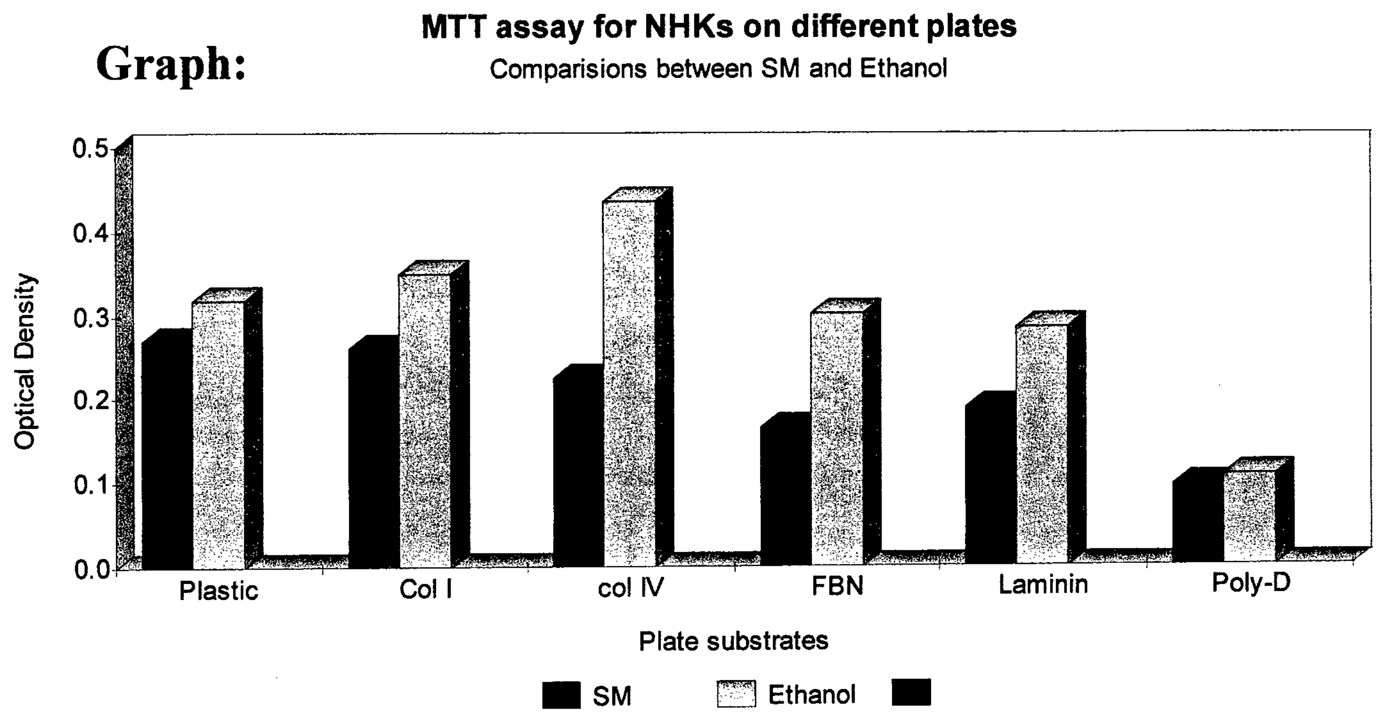


# Fig.10: MTT Assay for 50000NHKs on diff. Plates.

**Table:**

| | Plastic | Col I | col IV | FBN | Laminin | Poly-D |
|---|---|---|---|---|---|---|
| **SM** | **0.27** | 0.261 | 0.226 | 0.165 | 0.189 | 0.096 |
| **Ethanol** | **0.317** | 0.349 | 0.436 | 0.301 | 0.285 | 0.107 |

**Graph:**

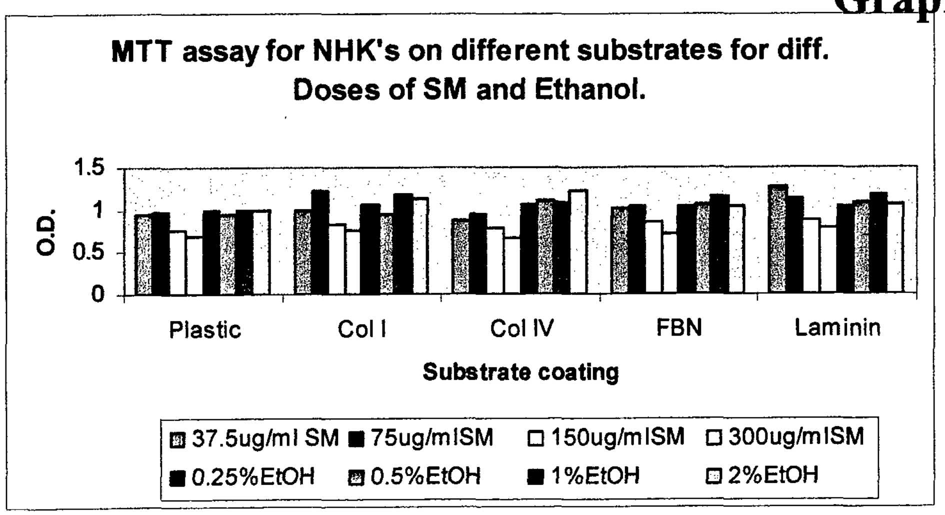


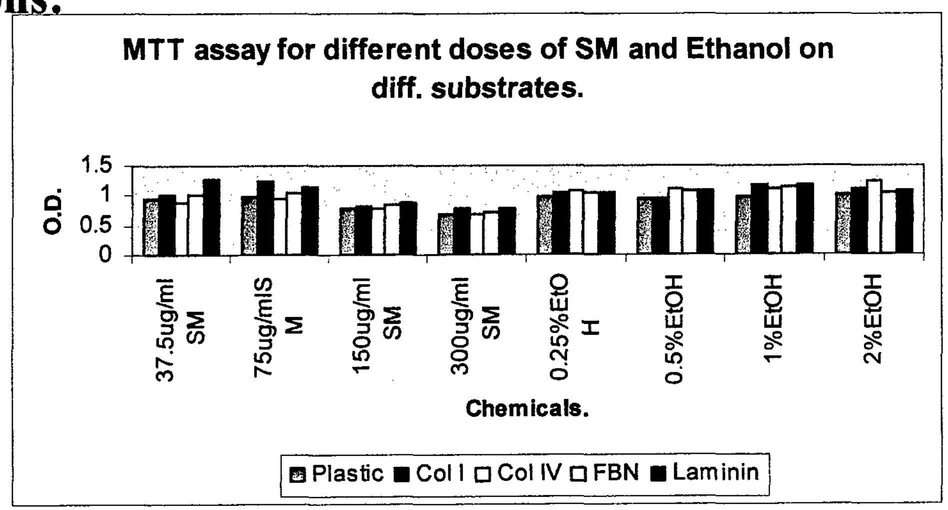
# Fig. 11: MTT Assay for different doses of SM and ethanol on diff. substrates.

**Table:**

| Doses/coating | Plastic | Col I | Col IV | FBN | Laminin |
|---|---|---|---|---|---|
| 37.5um of SM | 0.942 | 1.002 | 0.886 | 1.005 | 1.277 |
| 75um of SM | 0.98 | 1.225 | 0.95 | 1.032 | 1.133 |
| 150um of SM | 0.768 | 0.822 | 0.795 | 0.85 | 0.873 |
| 300um of SM | 0.701 | 0.769 | 0.679 | 0.71 | 0.786 |
| 0.25% EtOH | 0.991 | 1.059 | 1.067 | 1.038 | 1.044 |
| 0.5% EtOH | 0.957 | 0.951 | 1.103 | 1.06 | 1.083 |
| 1% EtOH | 0.992 | 1.178 | 1.096 | 1.155 | 1.173 |
| 2% EtOH | 0.996 | 1.123 | 1.228 | 1.048 | 1.068 |

**Graphs:**



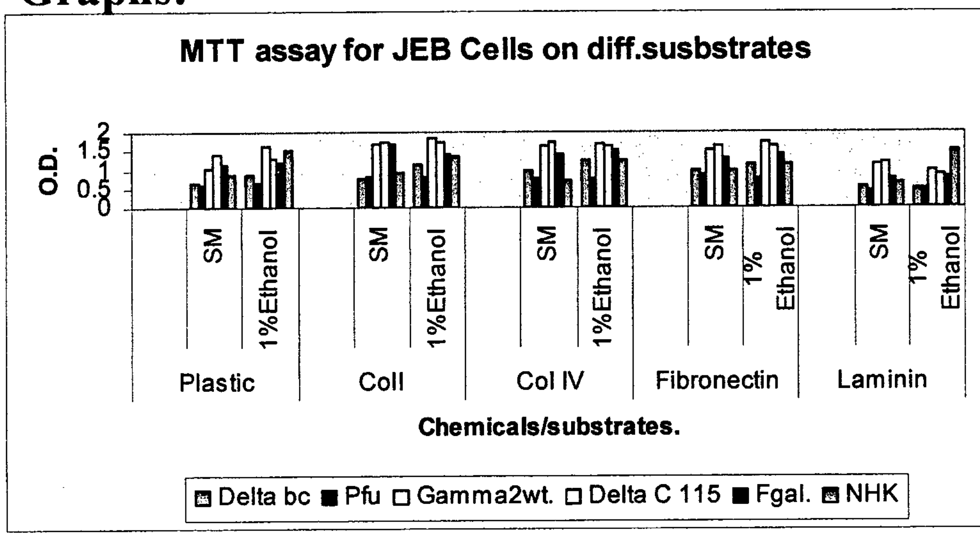



## Fig. 12: MTT assay for JEB Cells on diff. Substrates. (150um SM and 1% ethanol)

**Table:**

| Chemical/Substrates. | | Delta bc | Pfu | Gamma2wt. | Delta C 115 | Fgal. | NHK |
|---|---|---|---|---|---|---|---|
| Plastic | | | | | | | |
| | SM | 0.649 | 0.572 | 1.03 | 1.396 | 1.152 | 0.842 |
| | 1%Ethanol | 0.868 | 0.638 | 1.601 | 1.3 | 1.191 | 1.512 |
| Coll | | | | | | | |
| | SM | 0.749 | 0.836 | 1.673 | 1.74 | 1.674 | 0.931 |
| | 1%Ethanol | 1.153 | 0.797 | 1.823 | 1.724 | 1.408 | 1.327 |
| Col IV | | | | | | | |
| | SM | 0.996 | 0.75 | 1.645 | 1.73 | 1.416 | 0.687 |
| | 1%Ethanol | 1.253 | 0.765 | 1.676 | 1.639 | 1.531 | 1.268 |
| Fibronectin | | | | | | | |
| | SM | 0.97 | 0.842 | 1.497 | 1.599 | 1.318 | 0.973 |
| | 1% Ethanol | 1.143 | 0.757 | 1.707 | 1.595 | 1.402 | 1.149 |
| Laminin | | | | | | | |
| | SM | 0.526 | 0.418 | 1.141 | 1.198 | 0.766 | 0.658 |
| | 1% Ethanol | 0.494 | 0.475 | 0.954 | 0.852 | 0.833 | 1.499 |

**Graphs:**



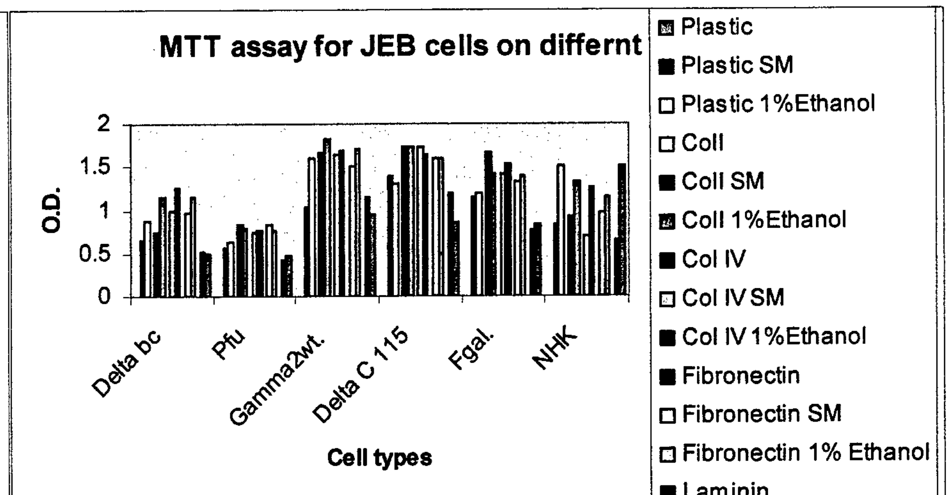
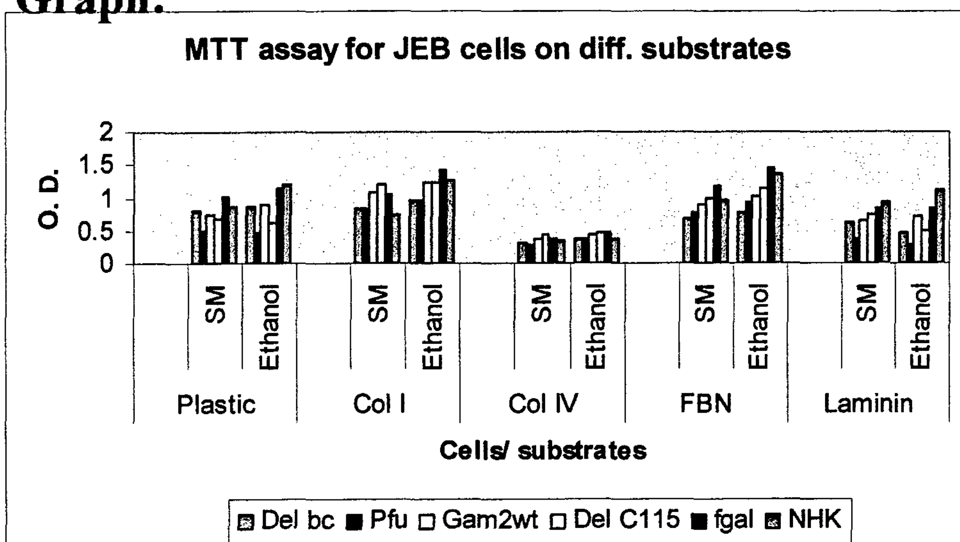



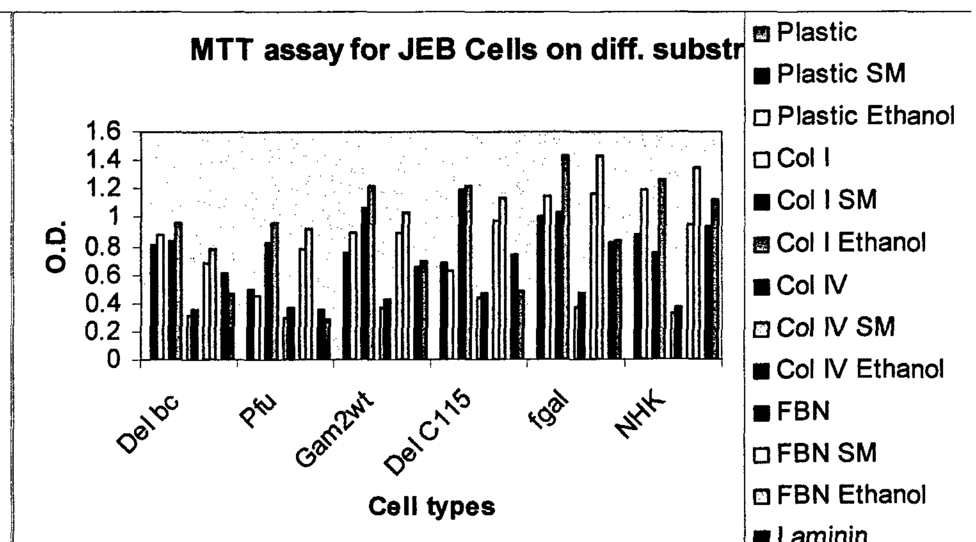
**Fig. 13: MTT Assay for diff. Cell types on diff. Substrates.(300*u*m SM & 2% Ethanol.**

**Table:**

| | | Del bc | Pfu | Gam2wt | Del C115 | fgal | NHK |
|---|---|---|---|---|---|---|---|
| Plastic | | | | | | | |
| | SM | 0.804 | 0.489 | 0.744 | 0.682 | 1.008 | 0.875 |
| | Ethanol | 0.875 | 0.447 | 0.885 | 0.63 | 1.151 | 1.187 |
| Col I | | | | | | | |
| | SM | 0.838 | 0.82 | 1.064 | 1.196 | 1.034 | 0.751 |
| | Ethanol | 0.956 | 0.968 | 1.219 | 1.224 | 1.429 | 1.263 |
| Col IV | | | | | | | |
| | SM | 0.316 | 0.291 | 0.373 | 0.441 | 0.369 | 0.328 |
| | Ethanol | 0.356 | 0.373 | 0.419 | 0.469 | 0.465 | 0.369 |
| FBN | | | | | | | |
| | SM | 0.677 | 0.78 | 0.885 | 0.971 | 1.162 | 0.944 |
| | Ethanol | 0.78 | 0.924 | 1.03 | 1.138 | 1.433 | 1.347 |
| Laminin | | | | | | | |
| | SM | 0.606 | 0.359 | 0.658 | 0.738 | 0.823 | 0.935 |
| | Ethanol | 0.474 | 0.285 | 0.697 | 0.479 | 0.831 | 1.114 |

**Graph:**



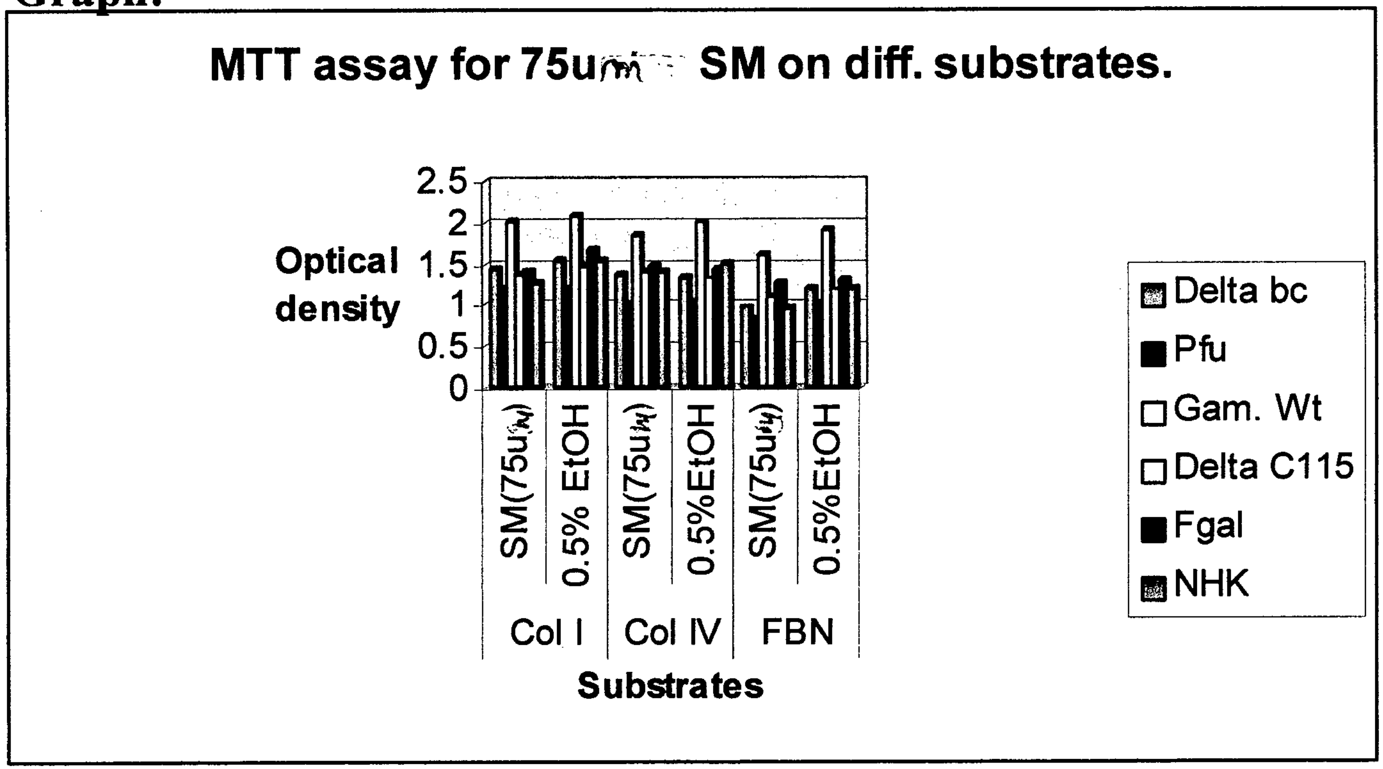



# Fig. 14: MTT assay for 75*u*m SM on diff. Substrates.

**Table:**

| Substrates | Chemicals | Delta bc | Pfu | Gam. Wt | Delta C115 | Fgal | NHK |
|---|---|---|---|---|---|---|---|
| Col I | SM(75um) | 1.433 | 1.219 | 2.021 | 1.376 | 1.421 | 1.265 |
| | 0.5% EtOH | 1.549 | 1.216 | 2.064 | 1.474 | 1.664 | 1.527 |
| Col IV | SM(75um) | 1.372 | 0.992 | 1.851 | 1.414 | 1.459 | 1.392 |
| | 0.5%EtOH | 1.33 | 1.039 | 1.99 | 1.318 | 1.448 | 1.512 |
| FBN | SM(75um) | 0.979 | 0.828 | 1.606 | 1.089 | 1.257 | 0.968 |
| | 0.5%EtOH | 1.201 | 0.997 | 1.898 | 1.184 | 1.306 | 1.193 |

**Graph:**

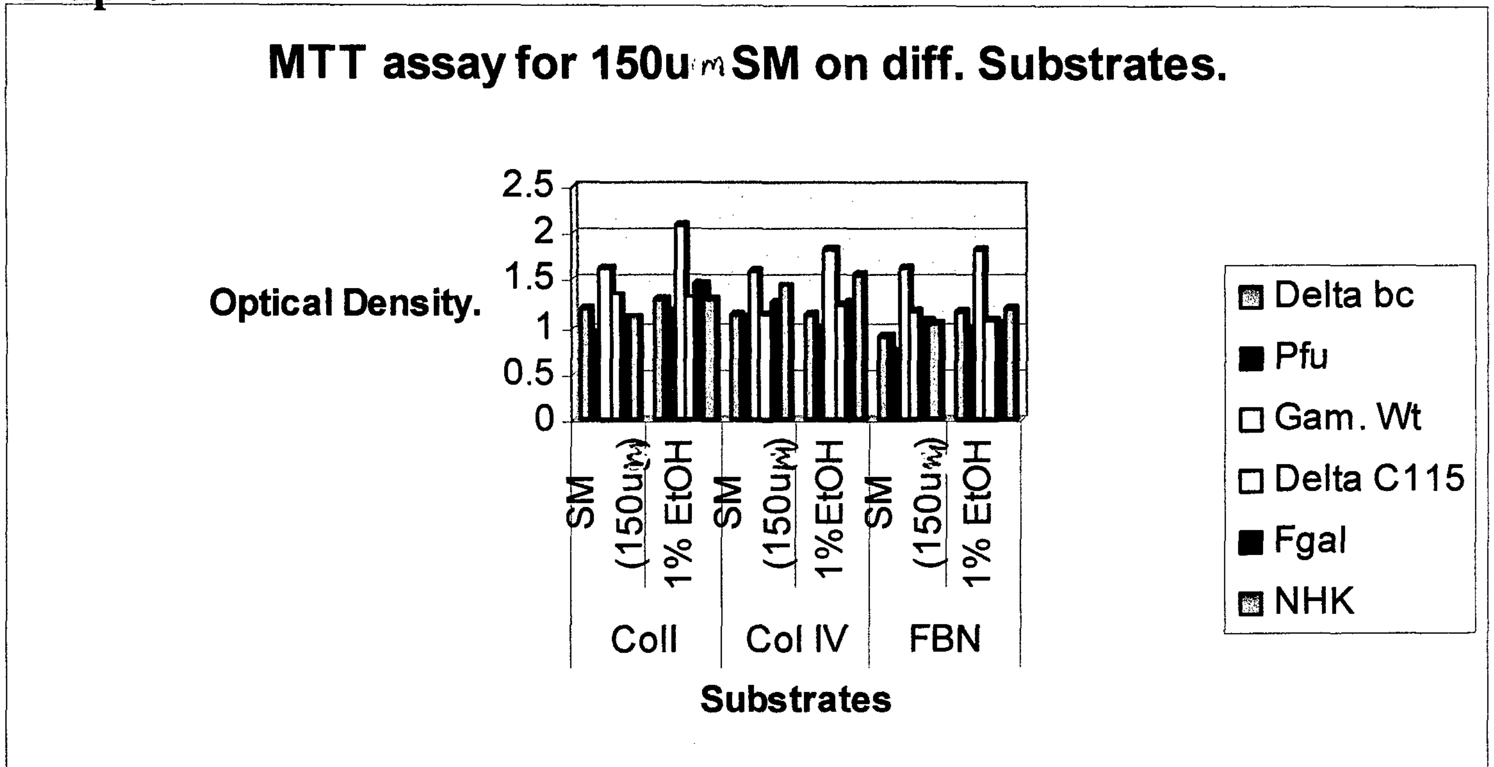


# Fig. 15: MTT assay for 150$u$m SM on diff. Substrates.

**Table:**

| Substrates | Chem. | Delta bc | Pfu | Gam. Wt | Delta C115 | Fgal | NHK |
|---|---|---|---|---|---|---|---|
| Coll | SM (150uM | 1.192 | 0.954 | 1.633 | 1.349 | 1.115 | 1.116 |
| | 1% EtOH | 1.288 | 1.156 | 2.095 | 1.315 | 1.455 | 1.309 |
| Col IV | SM (150uM | 1.126 | 1.094 | 1.59 | 1.134 | 1.258 | 1.446 |
| | 1%EtOH | 1.145 | 0.98 | 1.836 | 1.238 | 1.264 | 1.568 |
| FBN | SM (150uM | 0.907 | 0.739 | 1.627 | 1.158 | 1.068 | 1.03 |
| | 1% EtOH | 1.169 | 0.985 | 1.816 | 1.074 | 1.041 | 1.189 |

**Graph:**

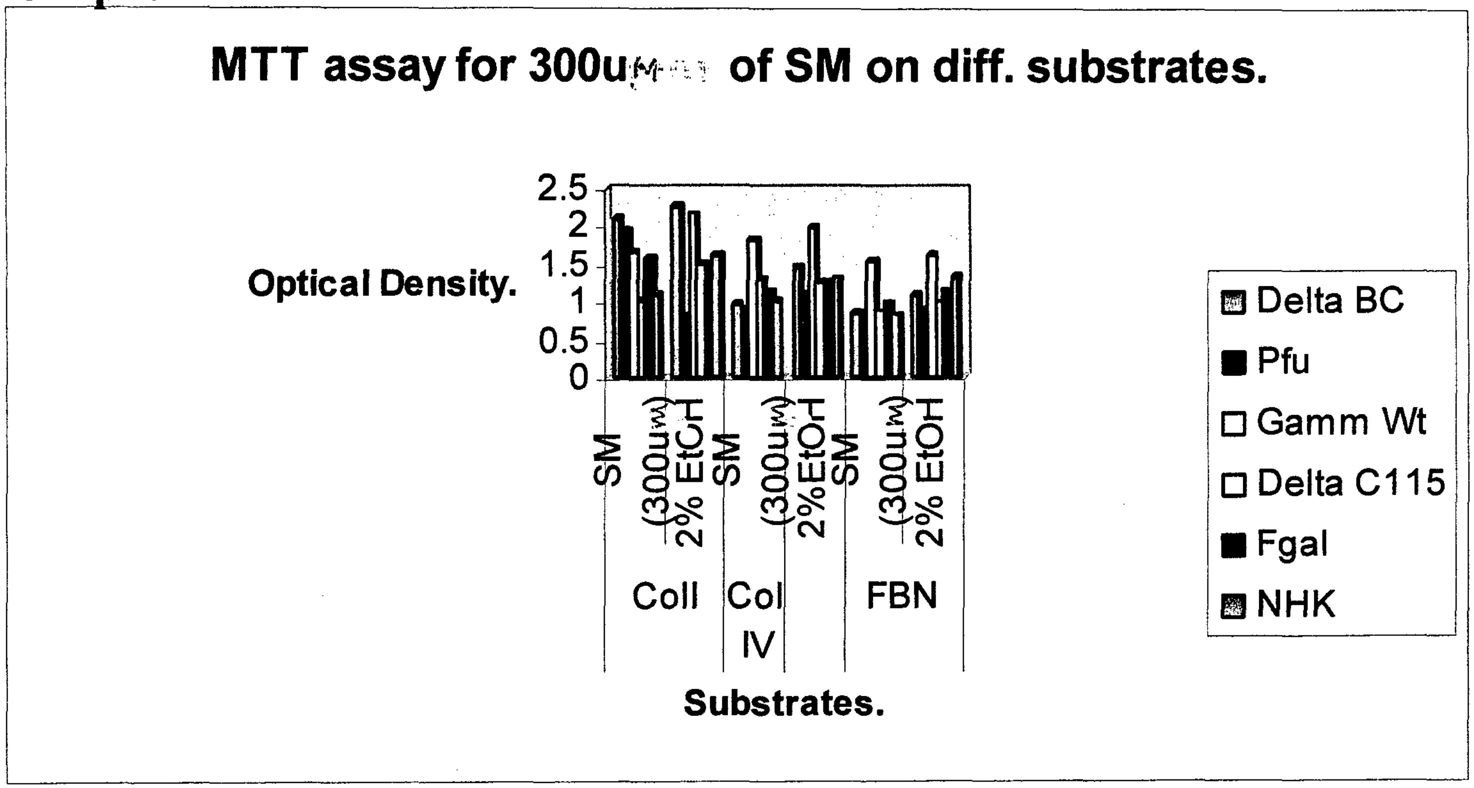


# Fig. 16: MTT Assay for 300*u*m SM on diff. Substrates.

**Table:**

| Sustrates | Chemicals | Delta BC | Pfu | Gamm Wt | Delta C115 | Fgal | NHK |
|---|---|---|---|---|---|---|---|
| Coll | SM (300um | 2.115 | 1.943 | 1.666 | 1.038 | 1.594 | 1.122 |
| | 2% EtOH | 2.277 | 0.828 | 2.178 | 1.514 | 1.433 | 1.637 |
| Col IV | SM (300um | 0.998 | 0.933 | 1.833 | 1.317 | 1.141 | 1.03 |
| | 2%EtOH | 1.478 | 1.112 | 2.002 | 1.273 | 1.295 | 1.326 |
| FBN | SM (300um | 0.876 | 0.594 | 1.538 | 0.892 | 1.007 | 0.846 |
| | 2% EtOH | 1.103 | 0.912 | 1.64 | 1.003 | 1.176 | 1.341 |

**Graph:**

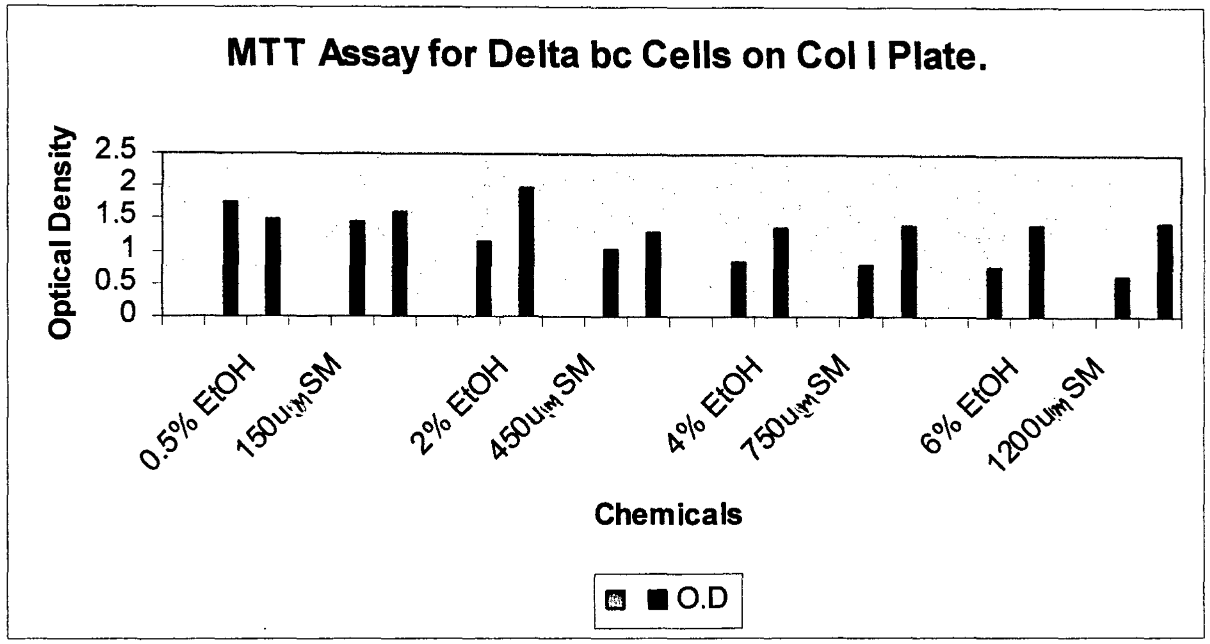


# Fig. 17: MTT Assay for Delta bc Cells on Col I Plate.

**Table:**

| Chemicals | O.D |
|---|---|
| 75uM SM | 1.745 |
| 0.5% EtOH | 1.491 |
| 150uM SM | 1.433 |
| 1% EtOH | 1.592 |
| 300uM SM | 1.119 |
| 2% EtOH | 1.978 |
| 450uM SM | 1.01 |
| 3% EtOH | 1.28 |
| 600uM SM | 0.82 |
| 4% EtOH | 1.352 |
| 750uM SM | 0.801 |
| 5% EtOH | 1.399 |
| 900uM SM | 0.767 |
| 6% EtOH | 1.394 |
| 1200uM SM | 0.593 |
| 8% EtOH | 1.428 |

**Graph:**

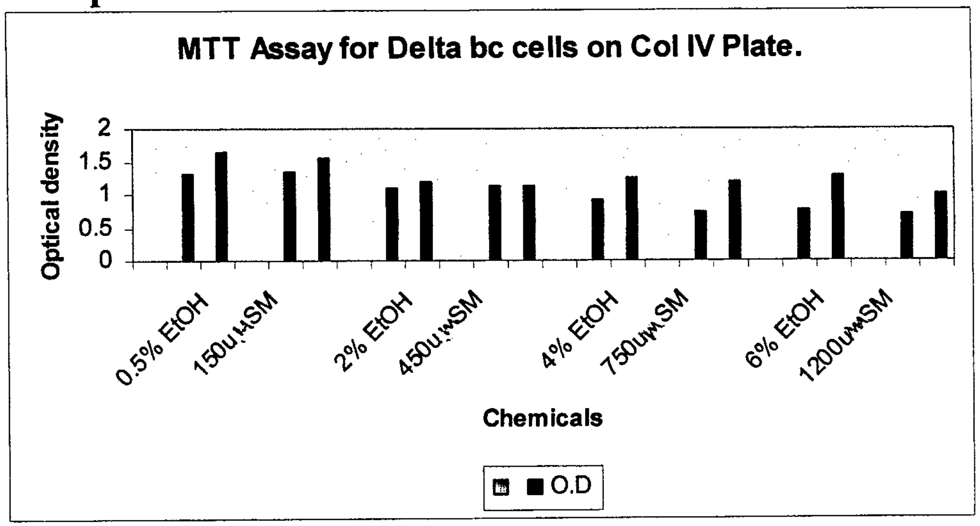


# Fig. 18: MTT Assay for Delta bc Cells on Col IV Plate.

**Table:**

| Chemicals | O.D |
|---|---|
| 75uM SM | 1.292 |
| 0.5% EtOH | 1.628 |
| 150uM SM | 1.32 |
| 1% EtOH | 1.539 |
| 300uM SM | 1.103 |
| 2% EtOH | 1.168 |
| 450uM SM | 1.112 |
| 3% EtOH | 1.124 |
| 600uM SM | 0.909 |
| 4% EtOH | 1.234 |
| 750uM SM | 0.729 |
| 5% EtOH | 1.193 |
| 900uM SM | 0.743 |
| 6% EtOH | 1.267 |
| 1200uM SM | 0.701 |
| 8% EtOH | 1.01 |

**Graph:**

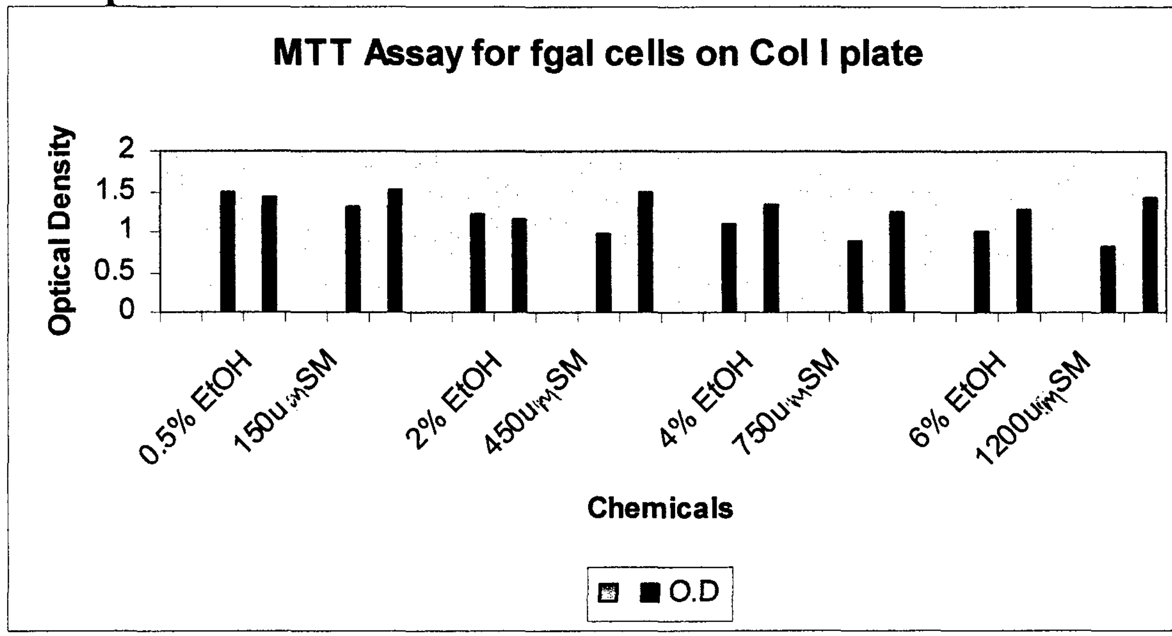


# Fig 19: MTT Assay for Fgal Cells on Col I Plate.

**Table:**

| Chemicals | O.D |
|---|---|
| 75uM SM | 1.49 |
| 0.5% EtOH | 1.427 |
| 150uM SM | 1.317 |
| 1% EtOH | 1.521 |
| 300uM SM | 1.202 |
| 2% EtOH | 1.164 |
| 450uM SM | 0.979 |
| 3% EtOH | 1.472 |
| 600uM SM | 1.078 |
| 4% EtOH | 1.334 |
| 750uM SM | 0.884 |
| 5% EtOH | 1.228 |
| 900uM SM | 1.003 |
| 6% EtOH | 1.284 |
| 1200uM SM | 0.82 |
| 8% EtOH | 1.436 |

**Graph:**

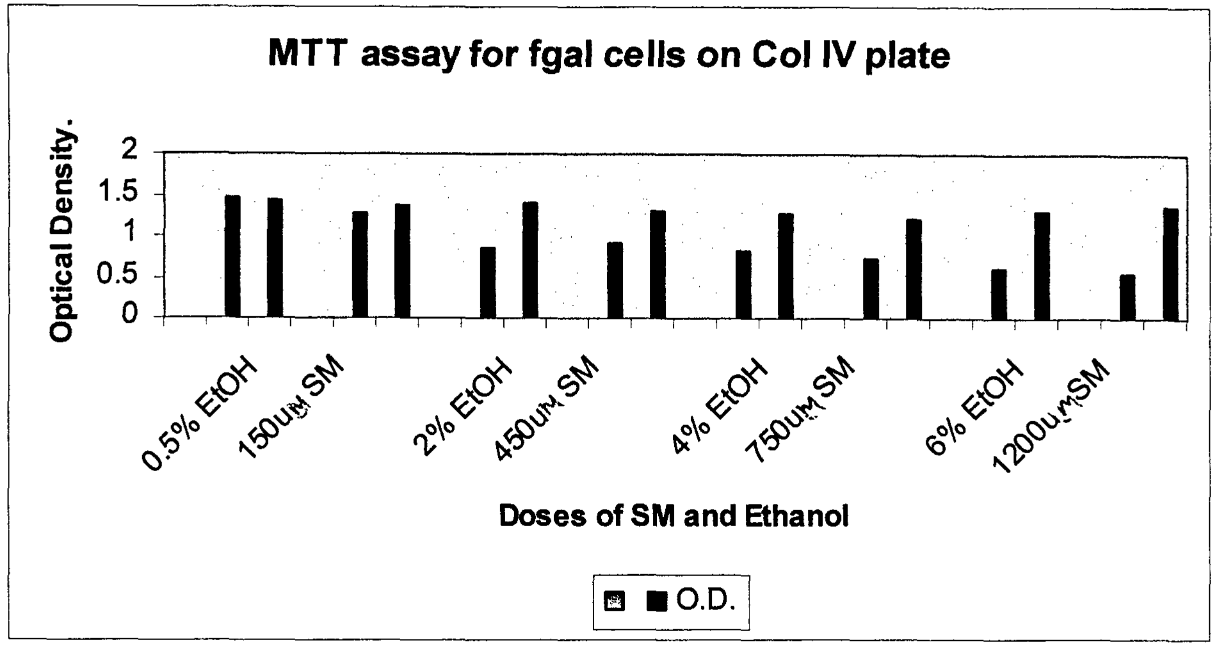


# Fig. 20: MTT Assay for Fgal cells on Col IV Plate.

**Table:**

| Chemicals | O.D. |
|---|---|
| 75um SM | 1.468 |
| 0.5% EtOH | 1.417 |
| 150um SM | 1.27 |
| 1% EtOH | 1.377 |
| 300um SM | 0.841 |
| 2% EtOH | 1.396 |
| 450um SM | 0.894 |
| 3% EtOH | 1.318 |
| 600um SM | 0.818 |
| 4% EtOH | 1.267 |
| 750um SM | 0.742 |
| 5% EtOH | 1.218 |
| 900um SM | 0.596 |
| 6% EtOH | 1.291 |
| 1200um SM | 0.544 |
| 8% EtOH | 1.377 |

**Graph:**



## Fig. 21: H & E Staining for Rafts exposed to SM and Ethanol.

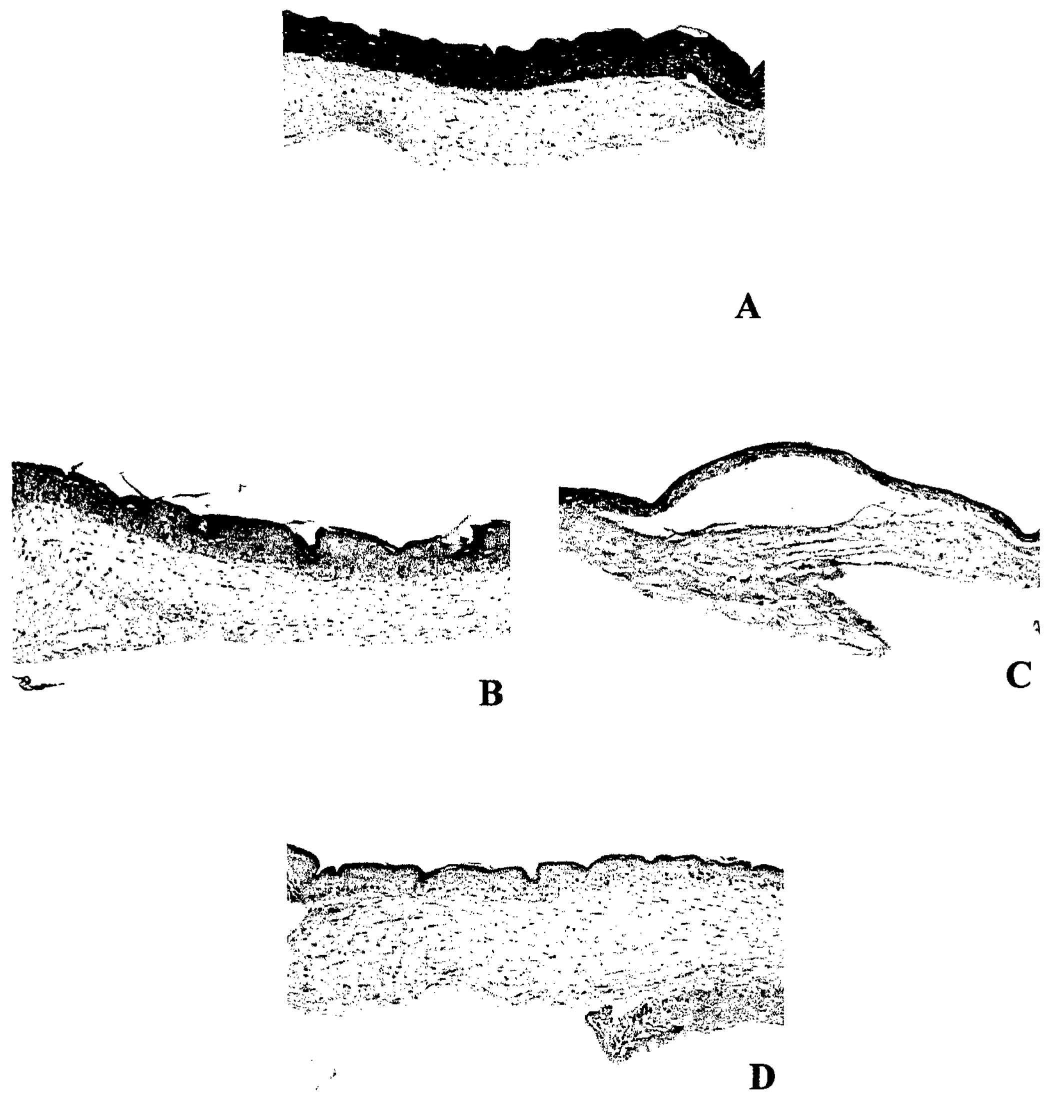



**Fig: A- Control; B & C – 75 & 150*u*m SM; D- 1% Ethanol.**

**Fig. 22: H&E Staining for Rafts exposed to diff. Doses of SM along with Controls**

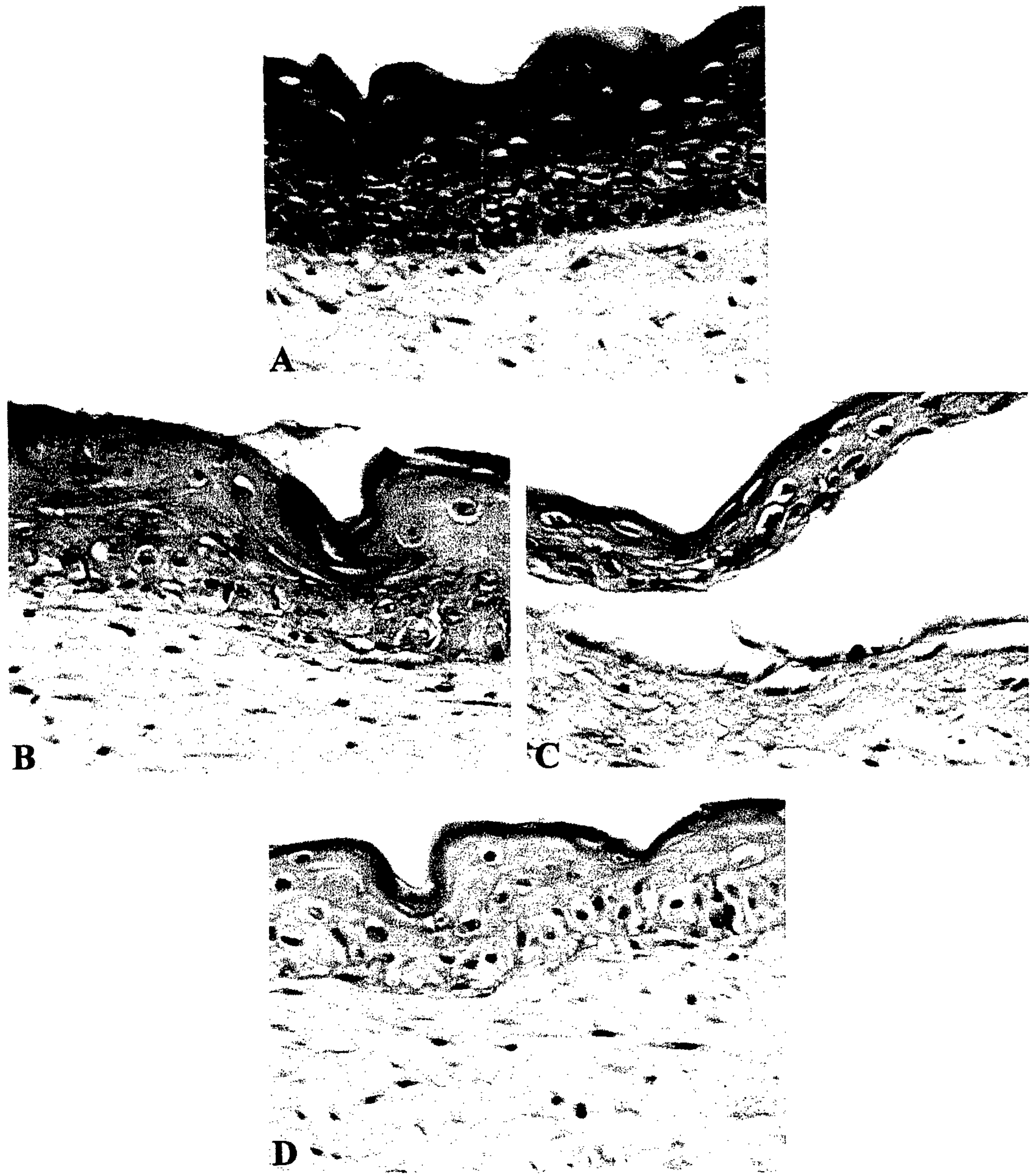



**Fig: A - Control; B & C – 75 & 150 *u*m SM; D - 1% Ethanol.**

**Fig. 23: M30 Staining for Rafts exposed to SM and Ethanol.**

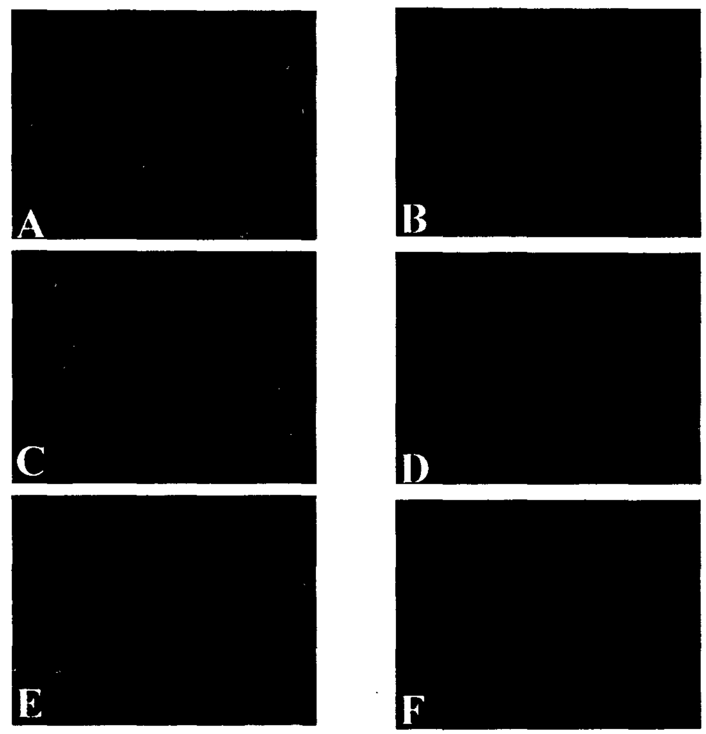



**Fig: A& B - Raft exposed to 1% Ethanol; C&D - Raft exposed to *75um* SM; E& F - Raft exposed to 150*u*m SM.**

A

## Fig. 24: H&E Staining for diff. doses of SM and Ethanol controls.

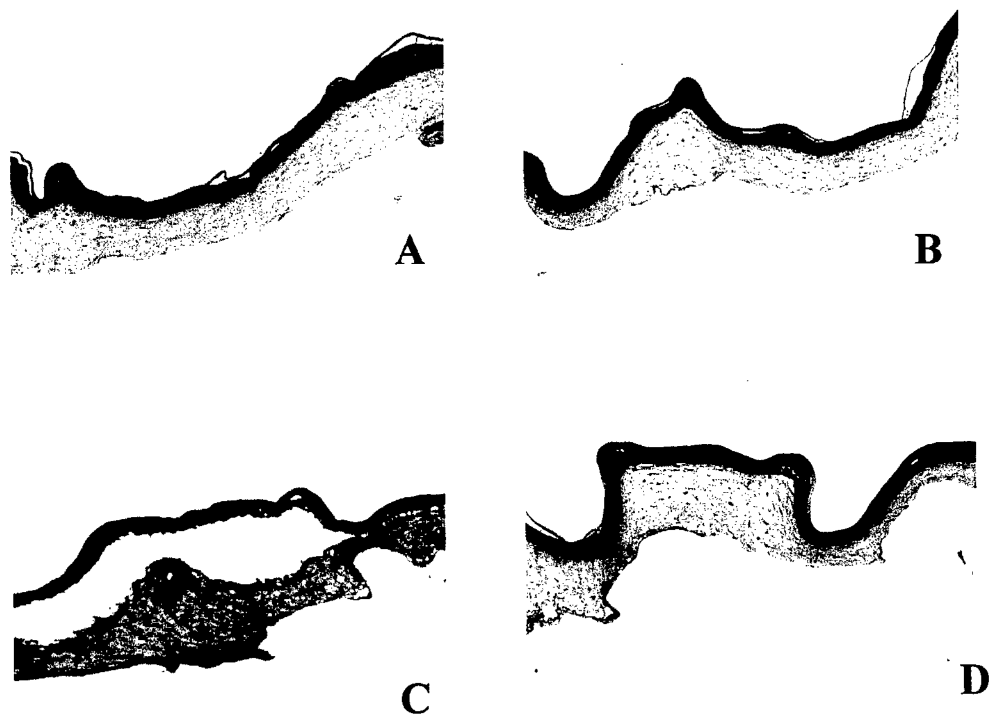



Fig : A & C – 150 &300*u*m of Sulfur Mustard.

B & D – 1 & 2 % Concurrent Ethanol controls.

## Fig. 25: H & E Staining for diff. Doses of SM and Ethanol.

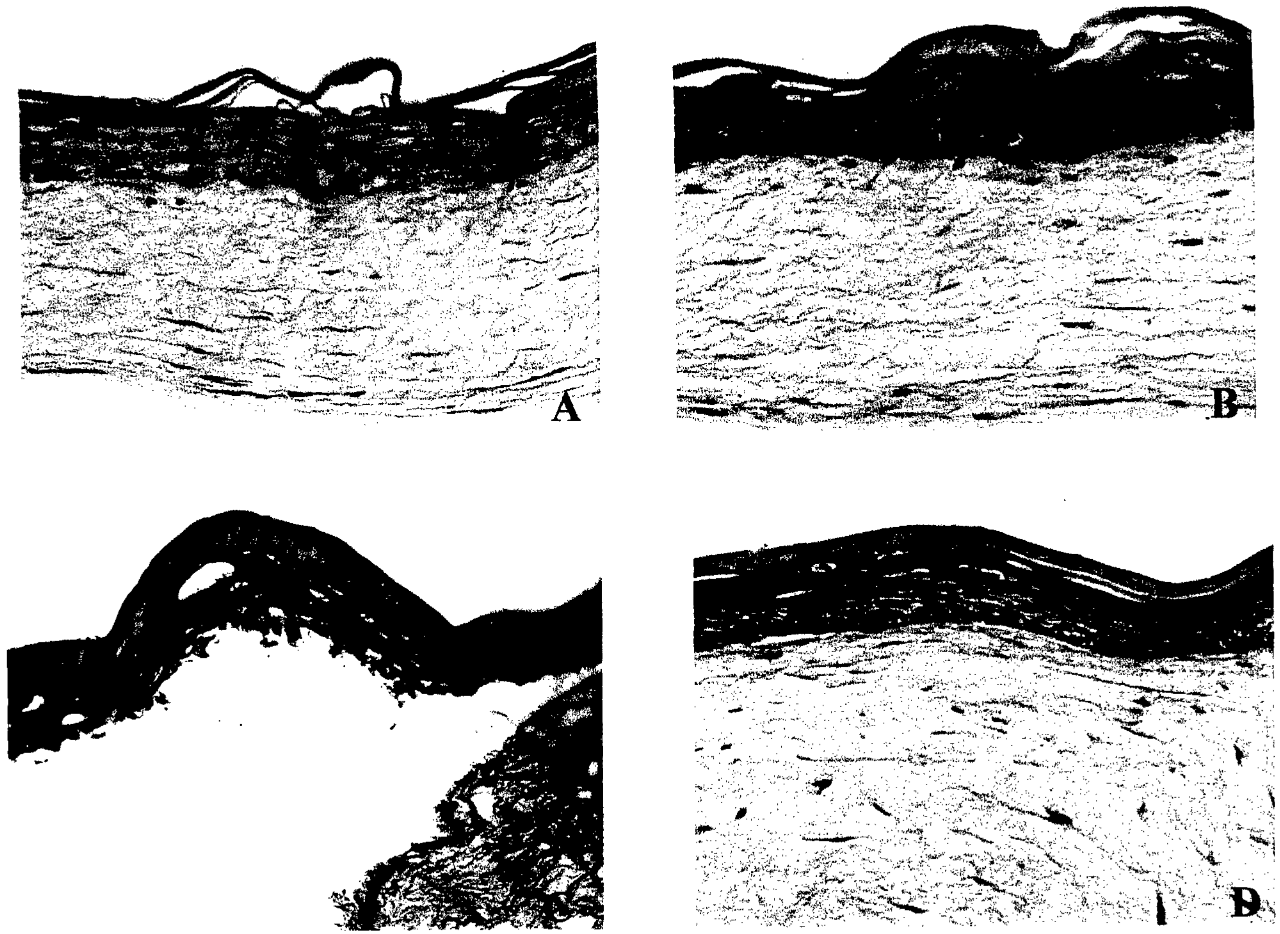
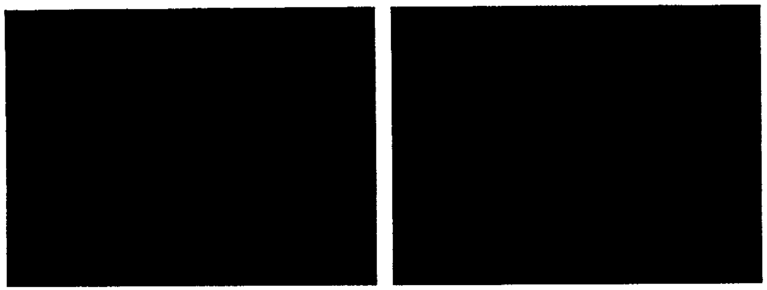



**Fig: A & C – 150 and 300*u*m of SM;**

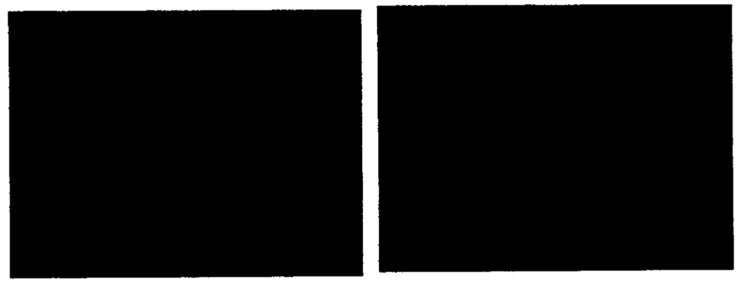
**B & C – 1 and 2 % Ethanol controls.**

Fig. 26: M30 Staining for the rafts exposed to diff . doses of SM and Ethanol

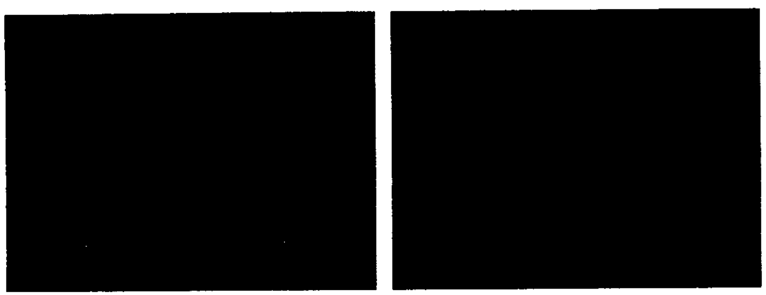
SM– 150um/7min.



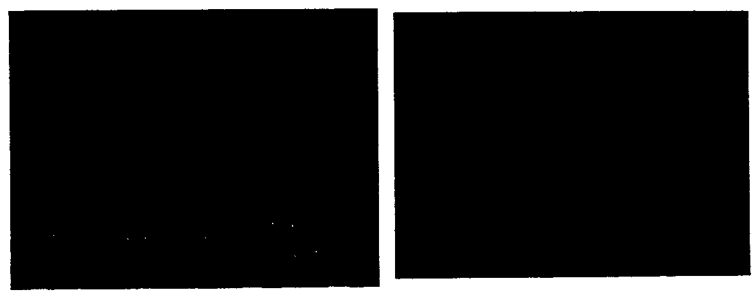
1% Ethanol/7min.



SM—300um/7min.



2% Ethanol/ 7min.



## Fig. 27: H&E Staining for NHK's on different substrates (3-D culture)

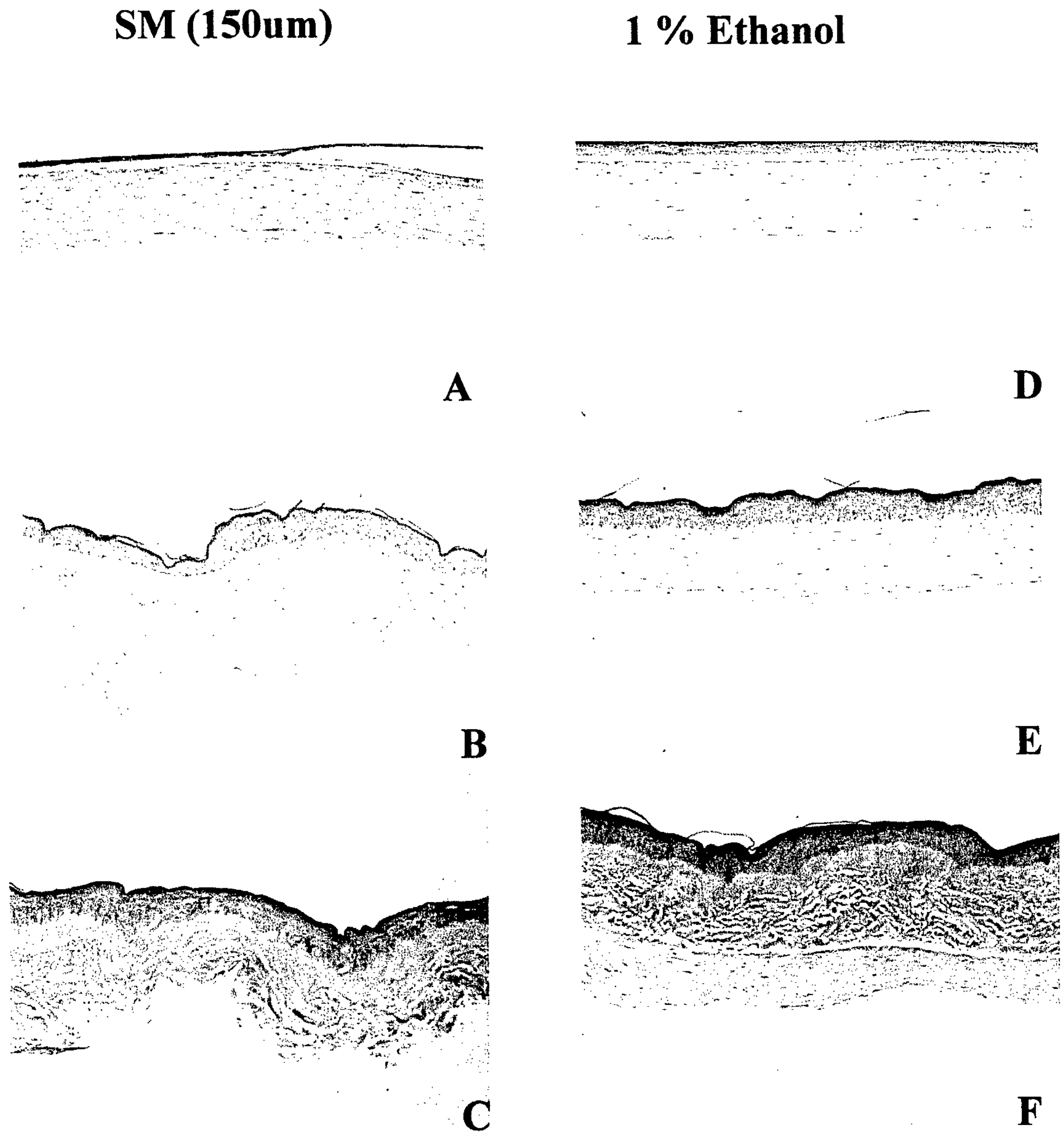



Fig: A&D- Plastic; B&E- Raft; C&F- Alloderm.

# Fig. 28: H & E Staining for NHK's on diff. substrates.

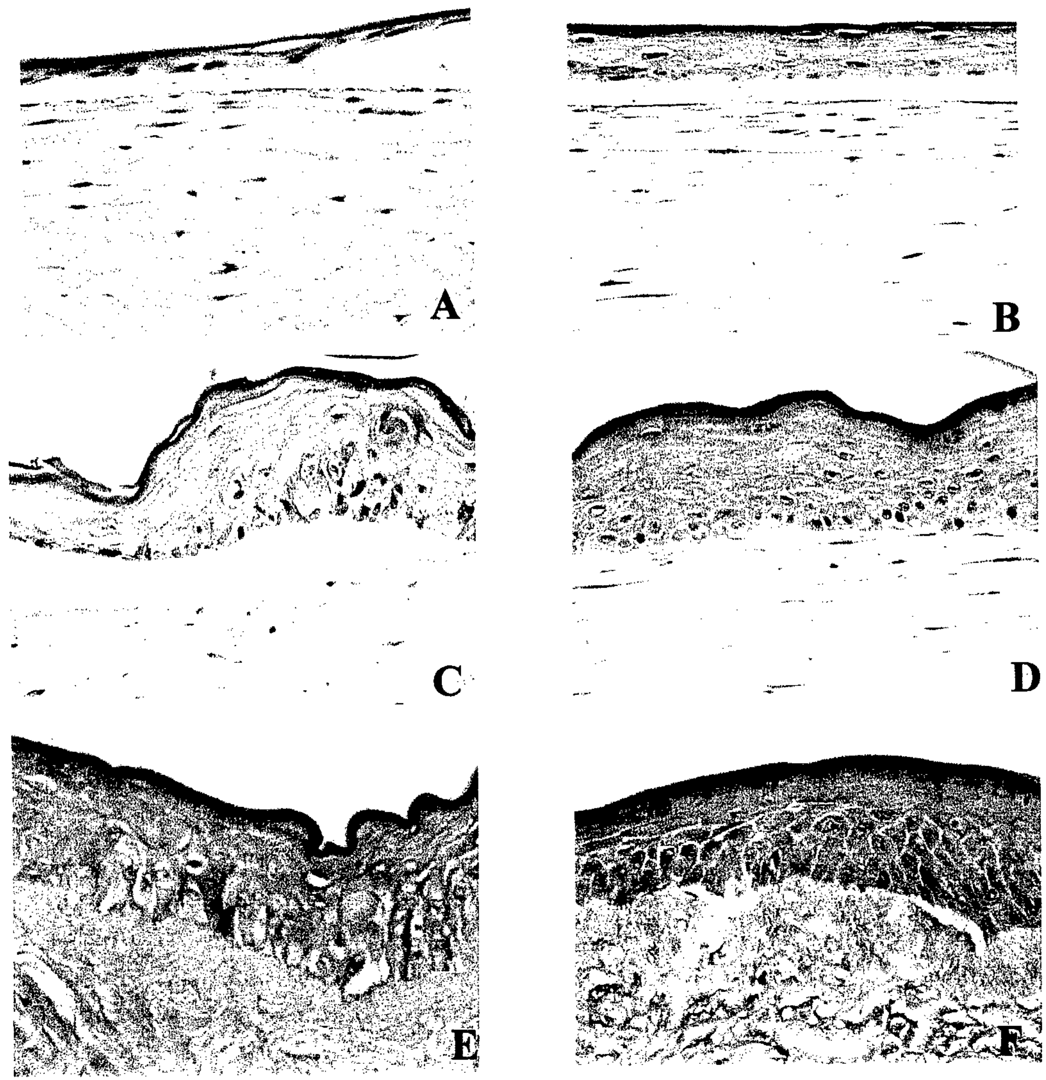



**Fig: A&B – Plastic; C&D – Raft; E&F - Alloderm.**

## Fig. 29: H & E Staining for NHK's on diff. Substrates (3- D cultures)

**SM (150*um*)** **1% Ethanol**

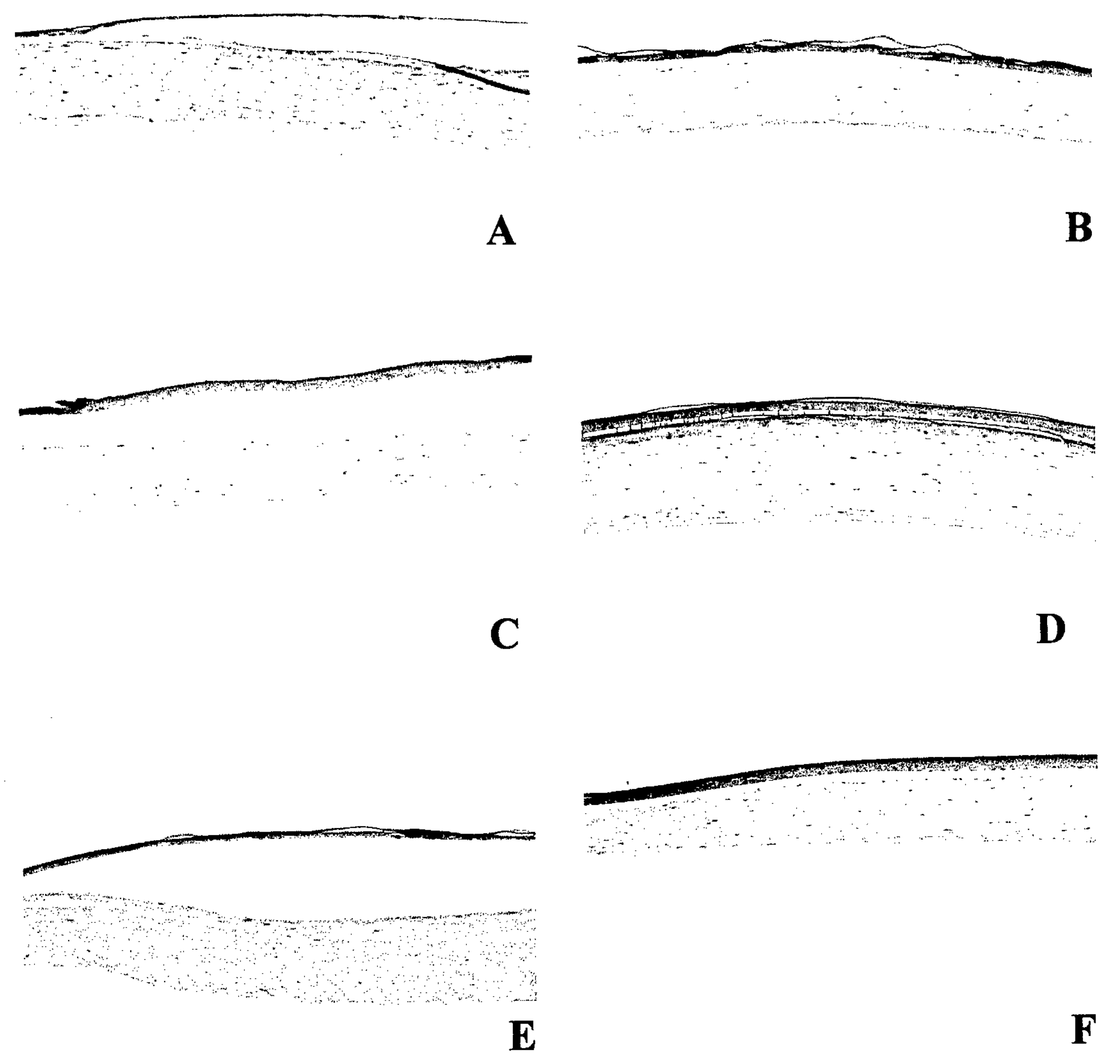



**Fig: A&B- Col I; C&D- Col IV; E& F- Fibronectin.**

# Fig. 30; Effects of SM and Ethanol on NHK's on diff. Substrates (3-D)

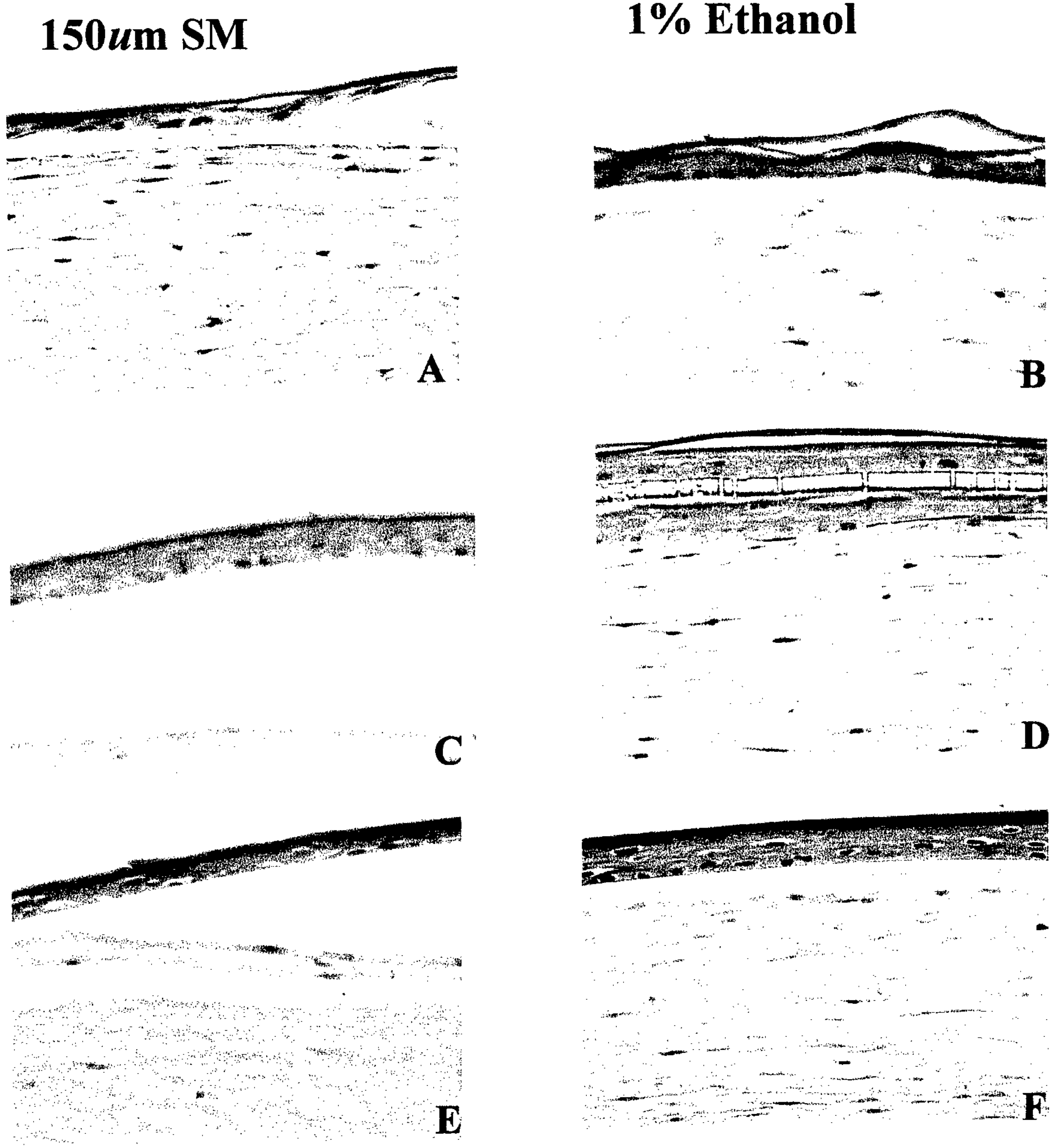



**Fig: A&B – Col I; C&D – Col IV; E&F – Fibronectin.**

## Fig. 31: Effects of SM on Alloderm and Raft along with controls.

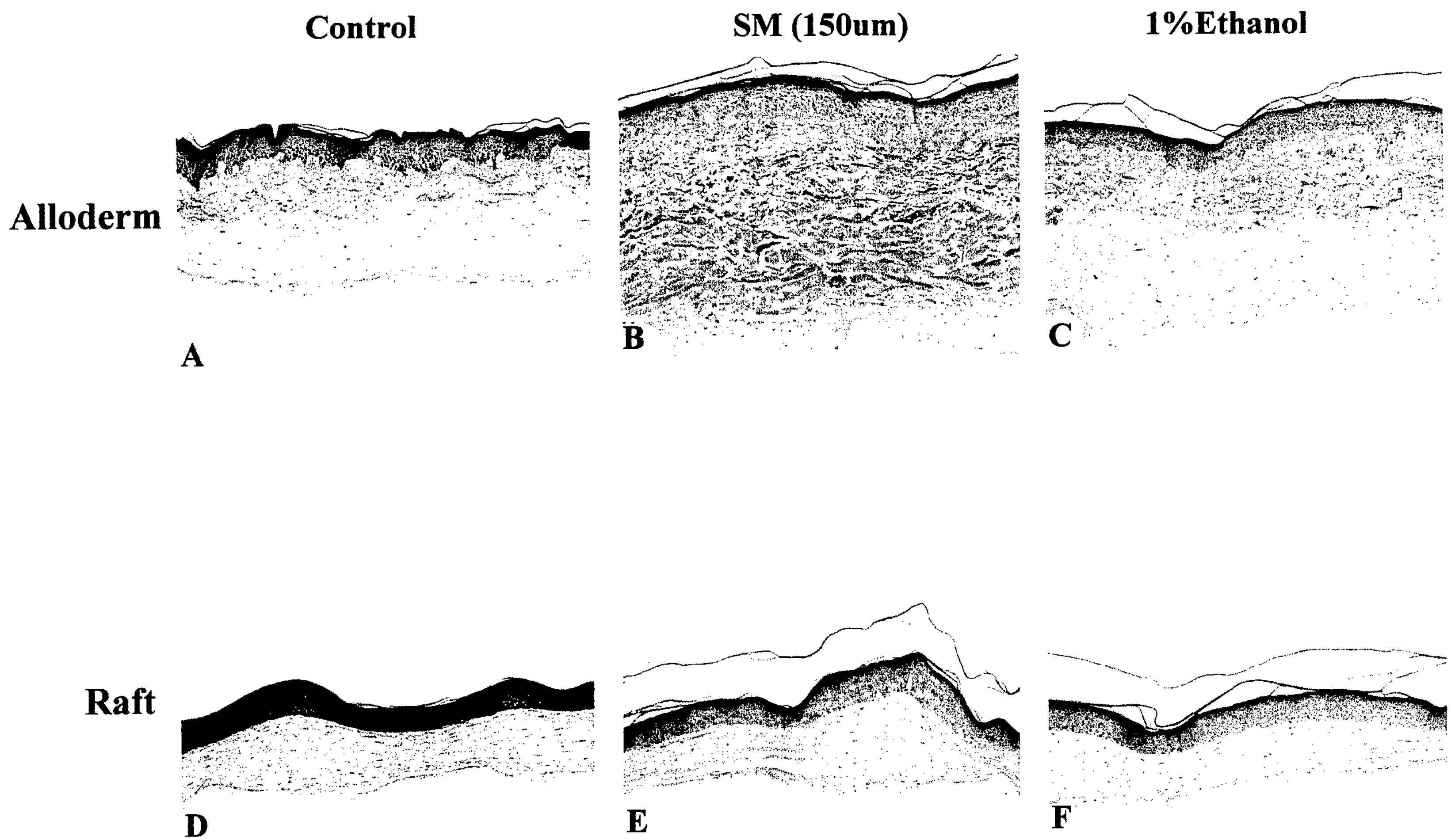



**Fig: A& D – Control; B& E – 150*u*m SM; C&F – 1% Ethanol.**

## Fig. 32: Effects of SM on Alloderm and Rafts along with controls.

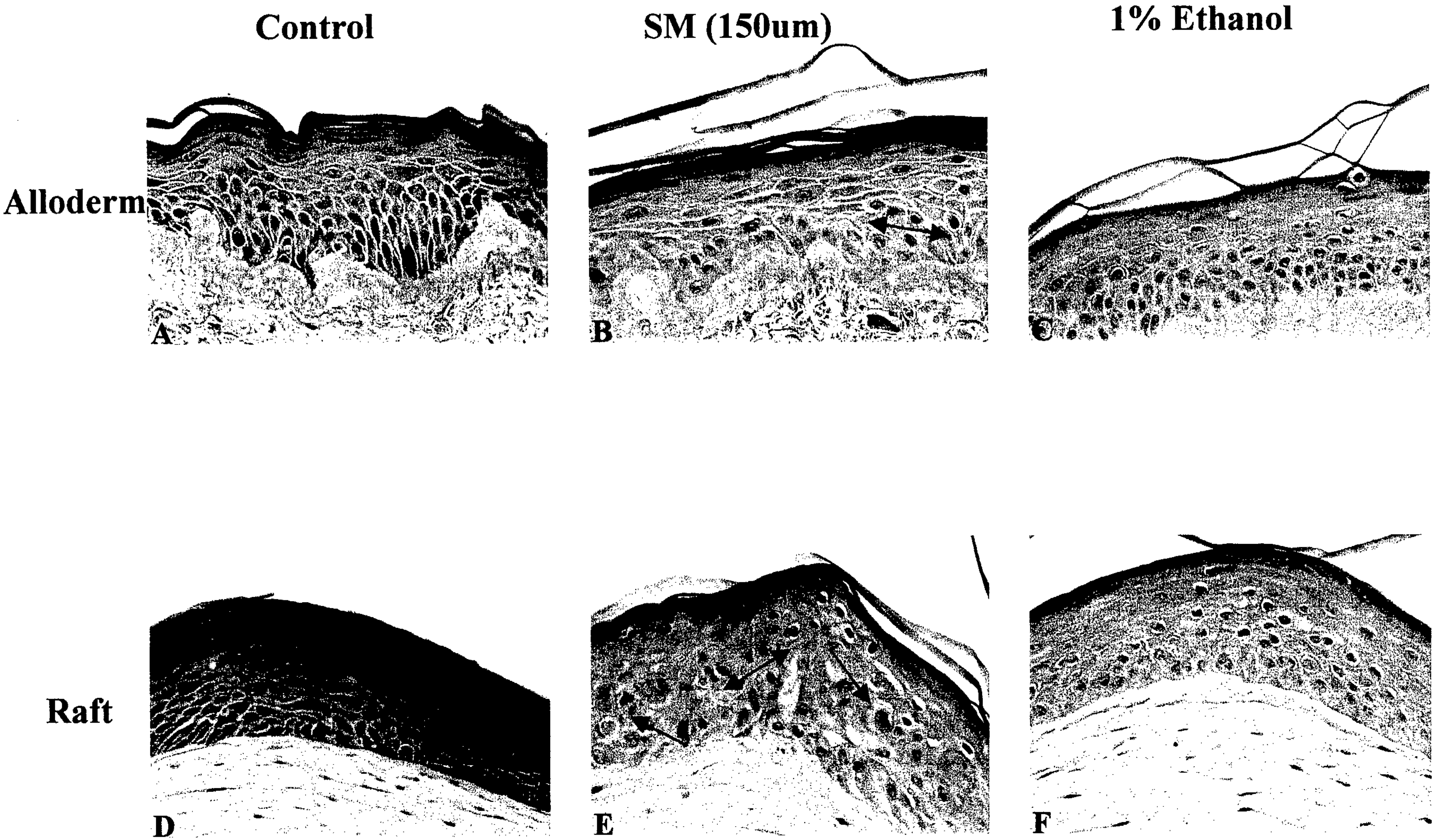
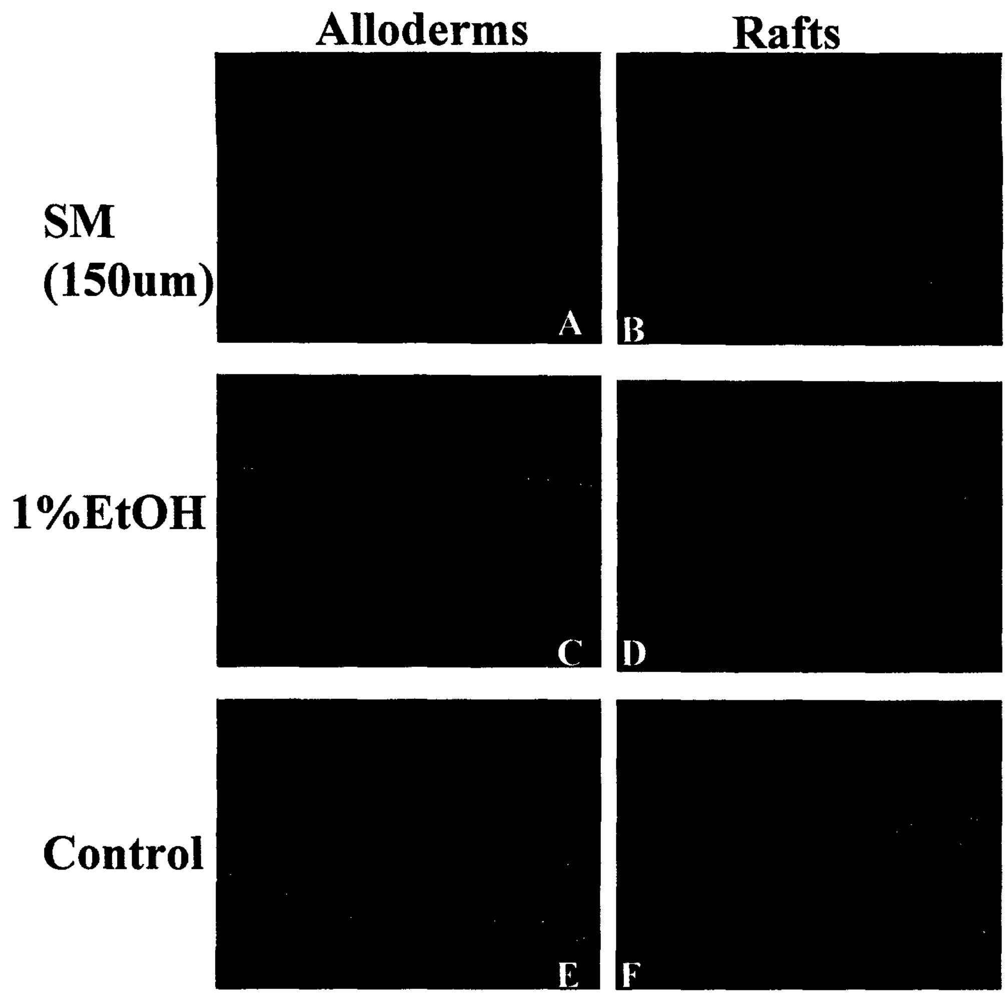



Fig: A&D – Controls; B&E - 150um of SM; C&F – 1% Ethanol.

Fig 33: M30 Staining for Alloderms and Rafts exposed to SM and Controls.



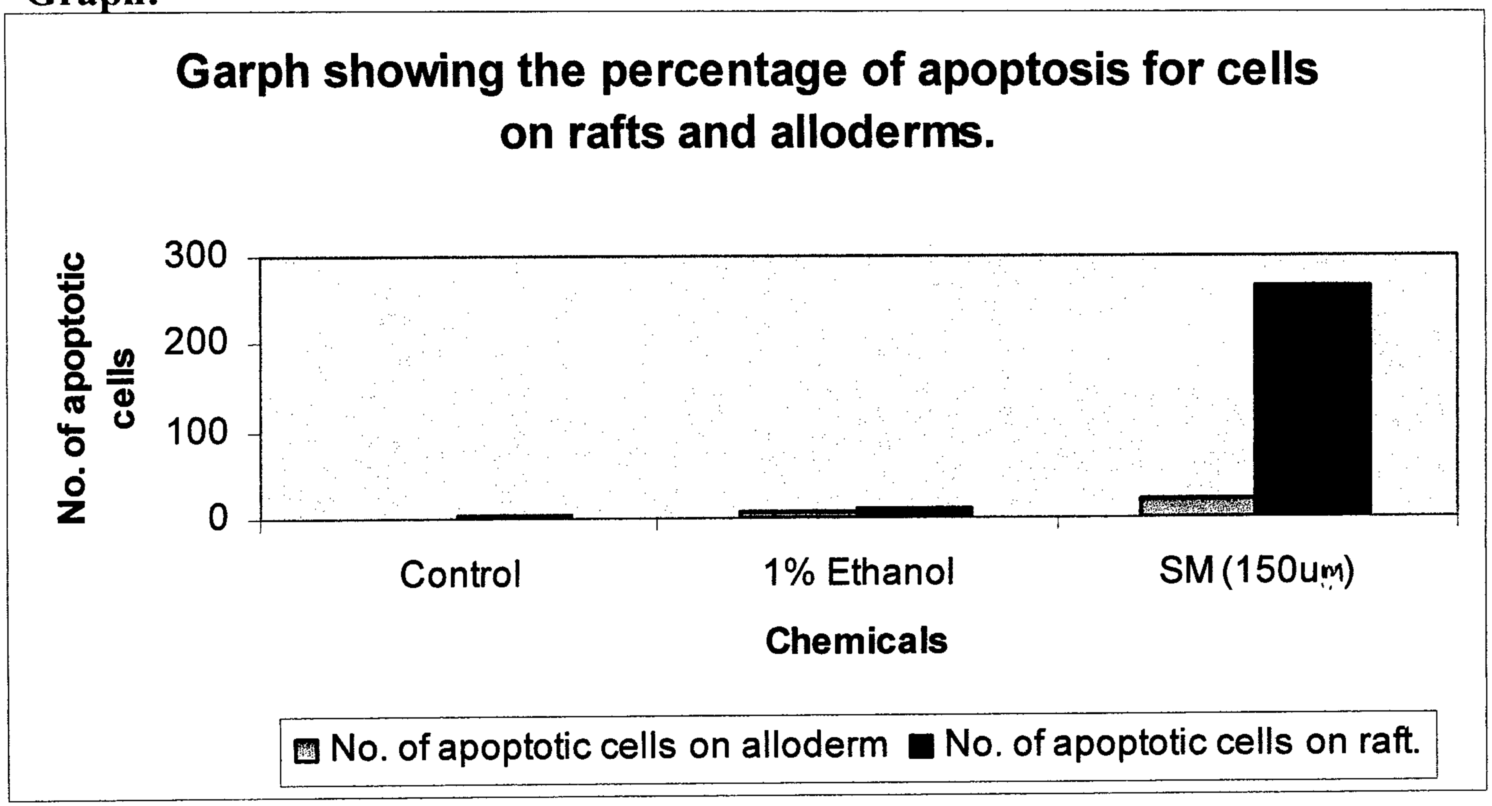

A,C, E –Alloderms; B, D, F- Rafts.

## Fig. 34: Apoptotic cell counts for keratinocytes on rafts and alloderms.

**Table:**

| Chemicals | No. of apoptotic cells on alloderm | No. of apoptotic cells on raft. |
|---|---|---|
| Control | 0 | 3 |
| 1% Ethanol | 6 | 9 |
| SM (150um) | 22 | 265 |

**Graph:**



## Fig. 35: Alloderm exposed to diff.doses of SM and Controls.

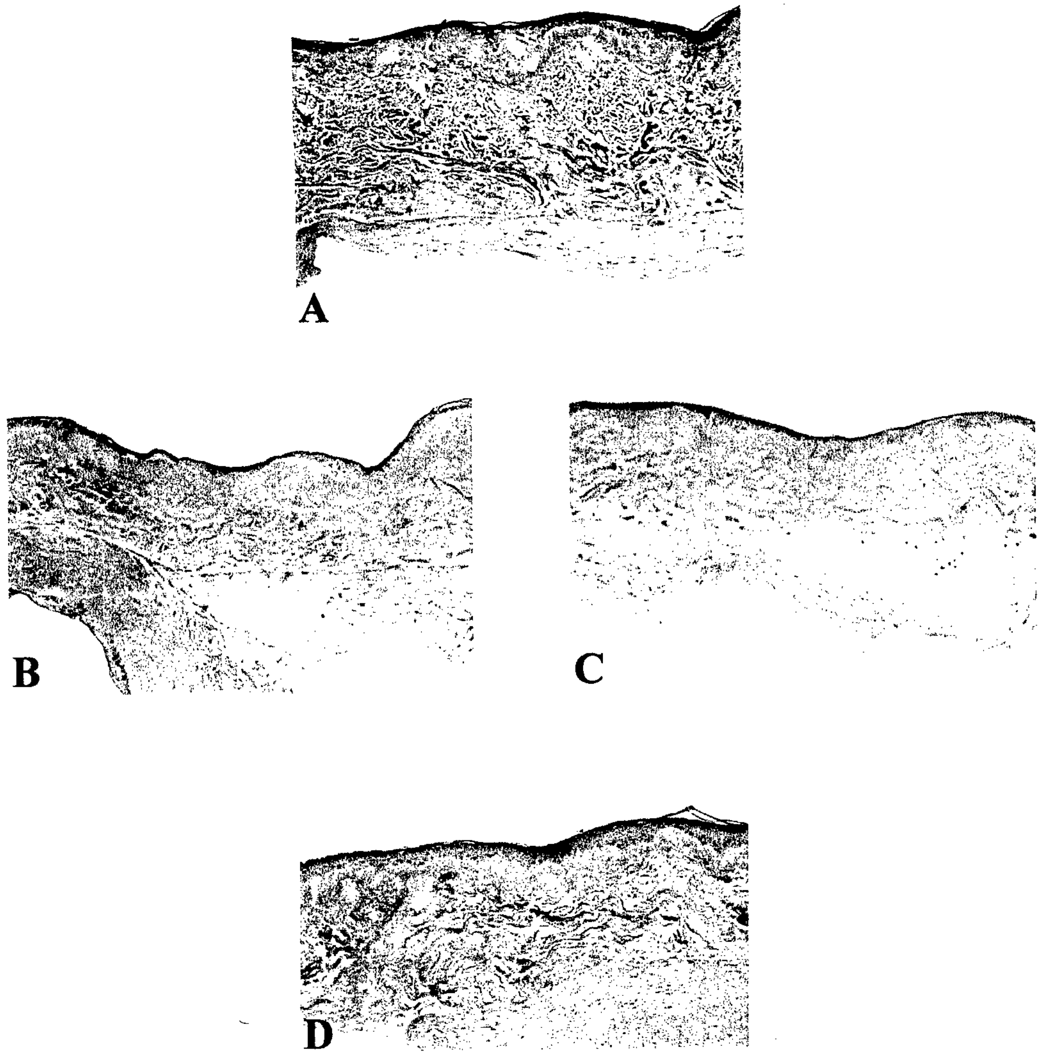



Fig: A-Control; B&C- 75& 150um SM; D- 1% Ethanol.

## Fig. 36: Alloderm exposed to diff. Doses of SM and Controls

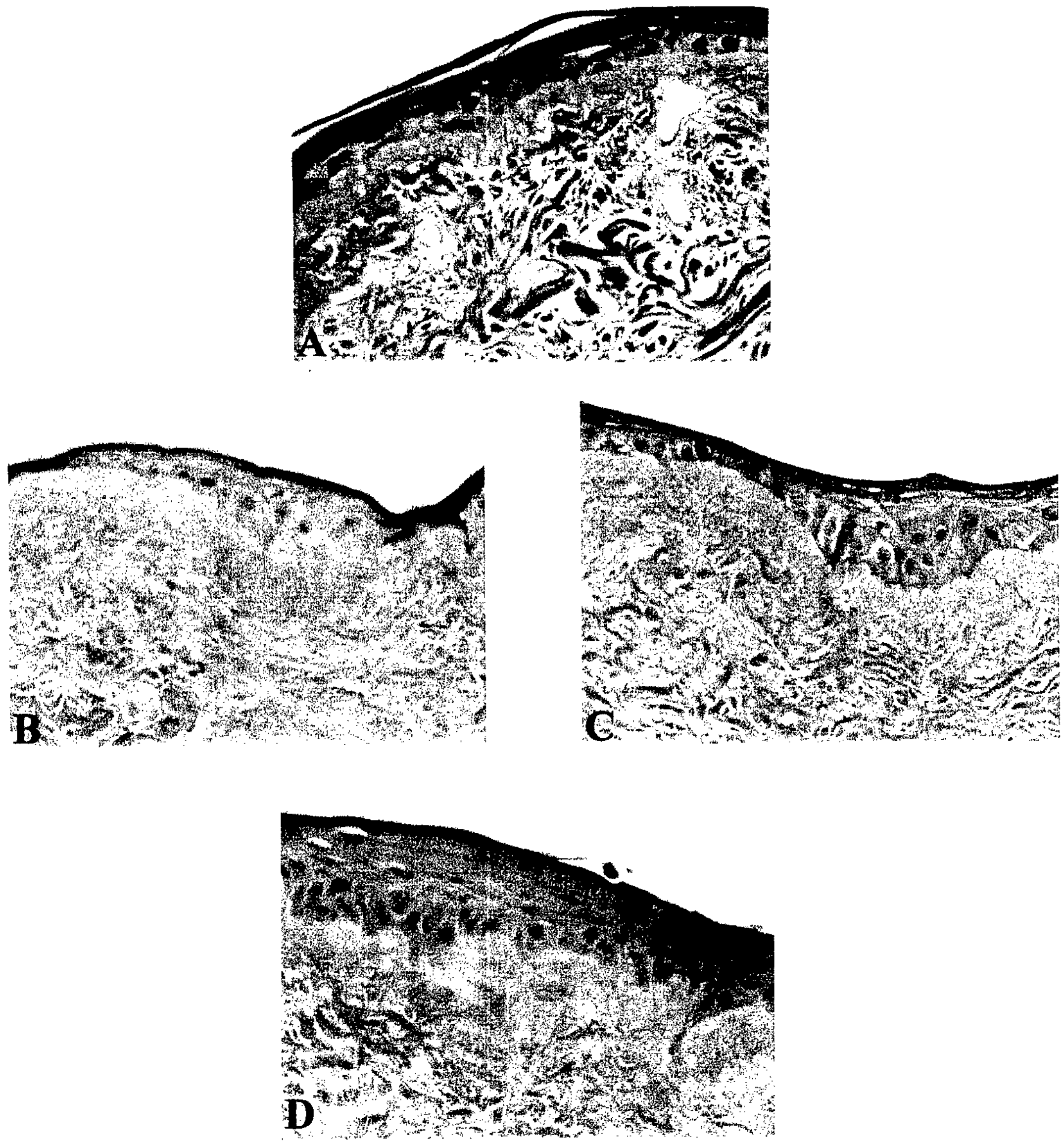



Fig: A- Control; B& C – 75 & 150um SM; D- 1% Ethanol.

**Fig. 37: M 30 Staining for Alloderm exposed to SM and Controls.**

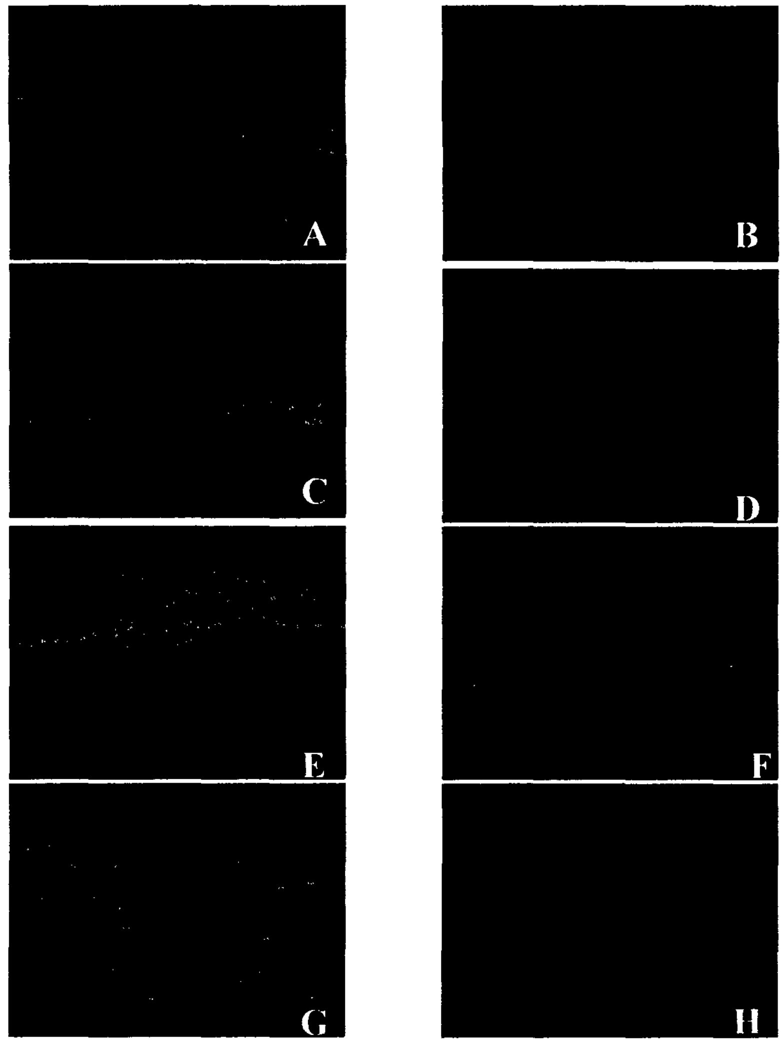



**Fig: A, B- Control; C, D- 75 um SM; E, F-150um SM; G, H- 1% Ethanol.**

# Fig. 38: Dose Response to SM shown by NHK's on Allos along with controls

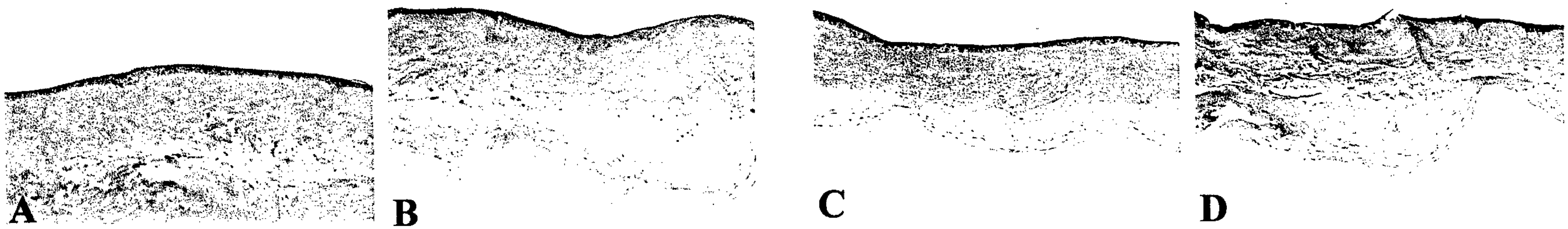
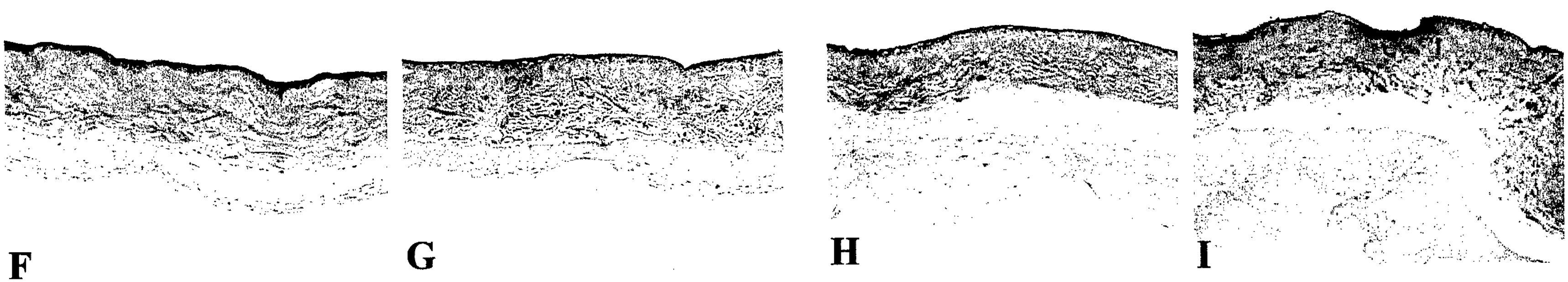




Fig: A-F – 75, 150, 300, 600, 1200um SM



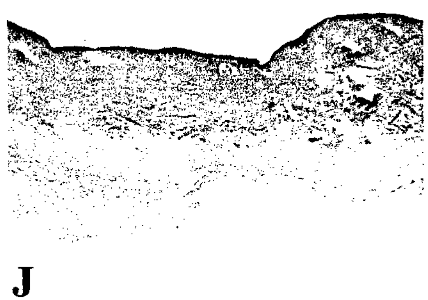




Fig: F-J- 0.5, 1, 2, 4, 8% Ethanol

## Fig.39: Dose Response to SM shown by NHK's on Alloderm along with controls

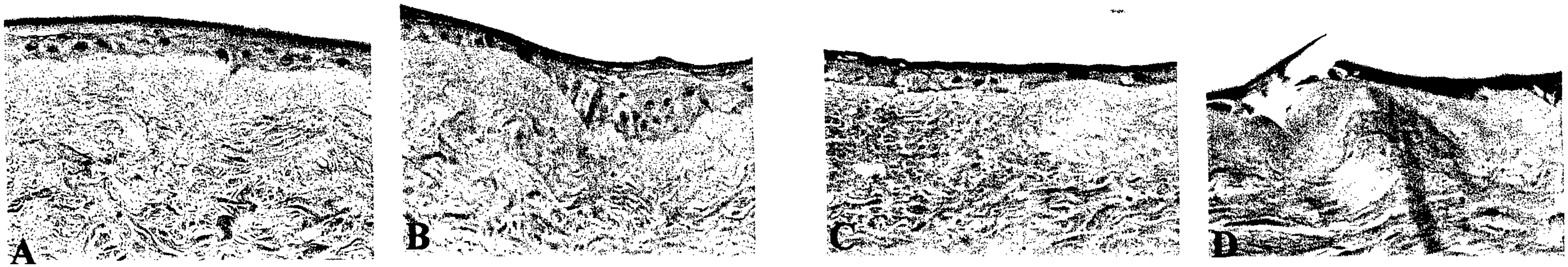
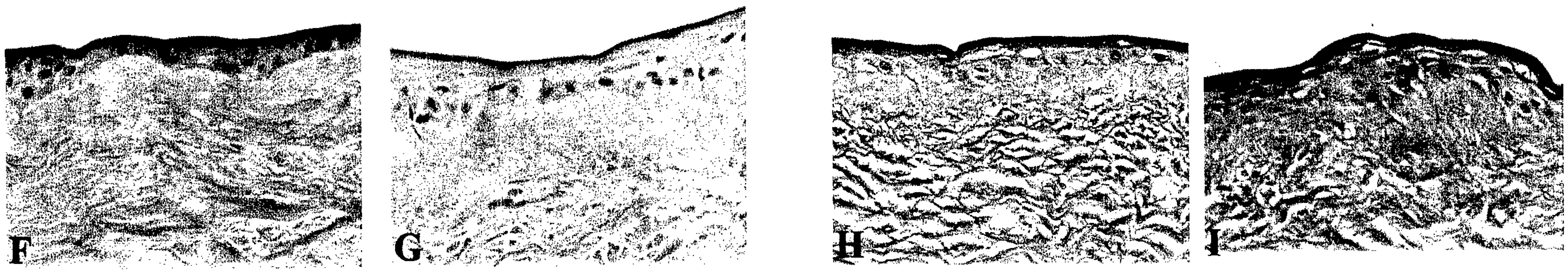




Fig: A-E – 75, 150, 300, 600, 1200um SM



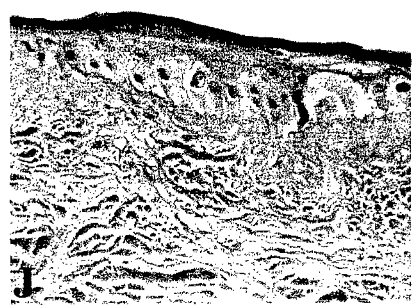




Fig: F-J – 0.5, 1, 2, 4, 8% Ethanol

## Fig. 40: M30 Staining for dose-response shown by NHKs on Alloderm

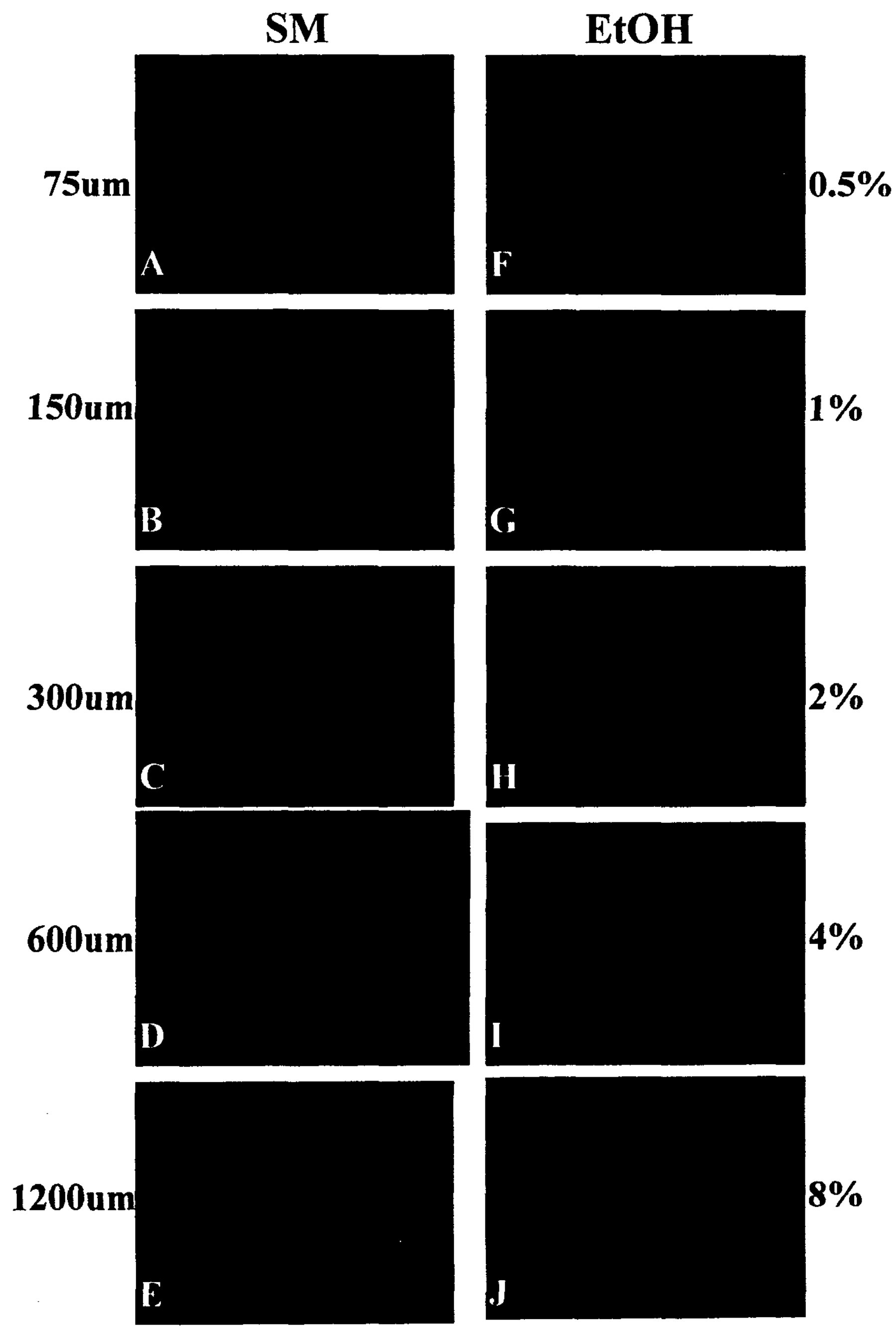


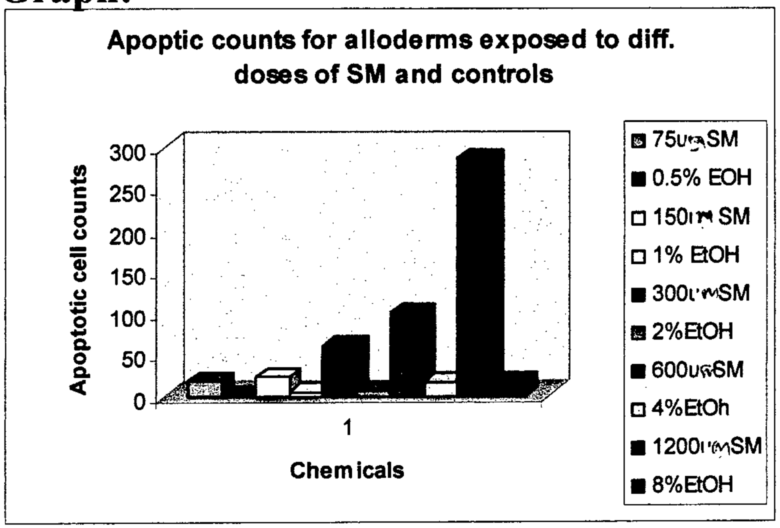

Fig: A-E – Diff. Doses of SM: F-J – Concurrent Ethanol controls.

## Fig. 41: Apoptotic Counts Alloderm Exposed to diff. Doses of SM along with concurrent controls

**Table:**

| Chemical | Apoptotic counts. |
|---|---|
| 75um SM | 19 |
| 0.5% EOH | 0 |
| 150um SM | 24 |
| 1% EtOH | 5 |
| 300um SM | 61 |
| 2%EtOH | 6 |
| 600umSM | 102 |
| 4%EtOH | 17 |
| 1200um SM | 286 |
| 8%EtOH | 18 |

**Graph:**



## Fig. 42: Effects of SM on diff. Cell types seeded on alloderm along with controls.

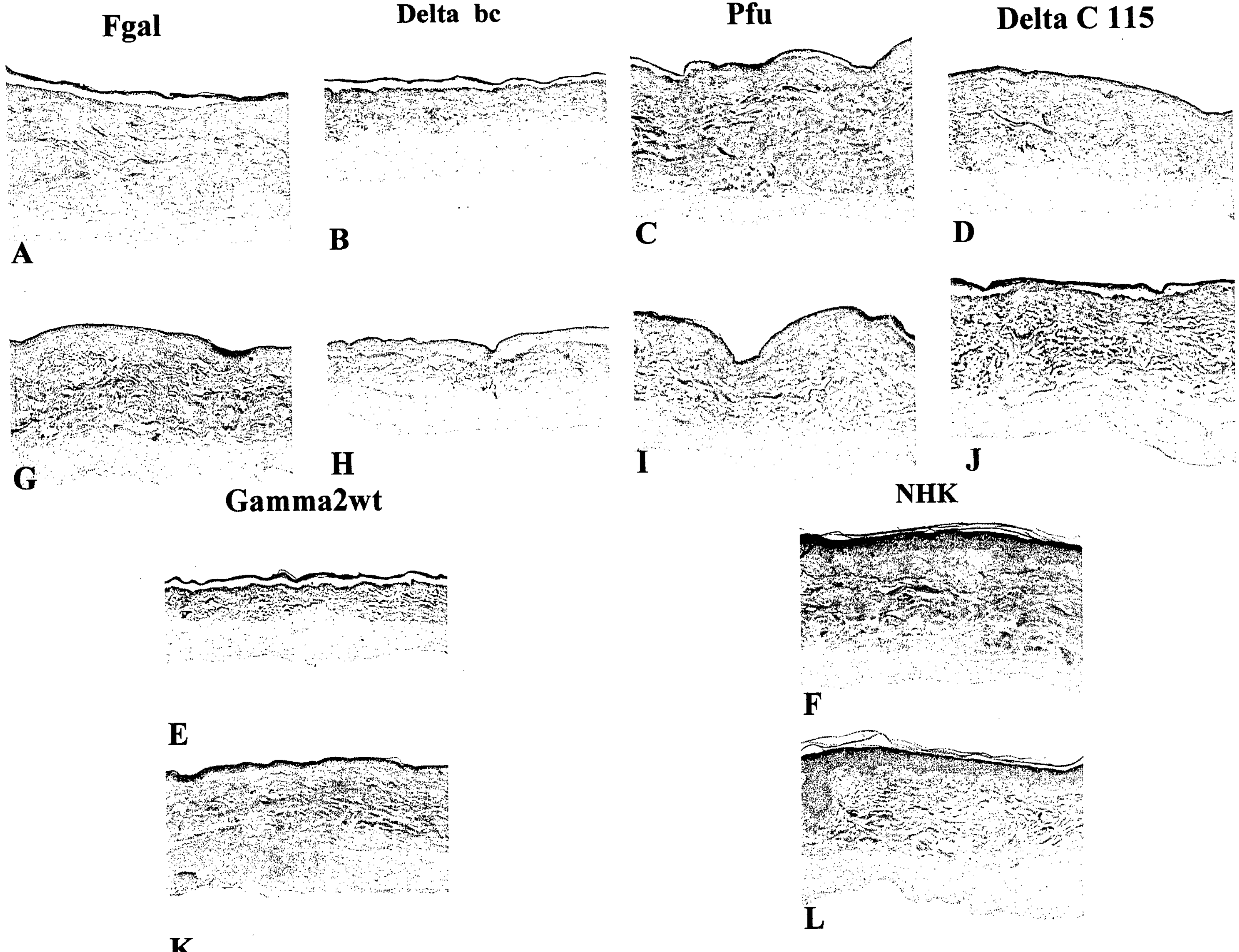



Fig: A-F – 150um of SM: G-I – 1% Ethanol.

# Fig. 43: Effects of SM on diff. Cell types on alloderm along with controls.

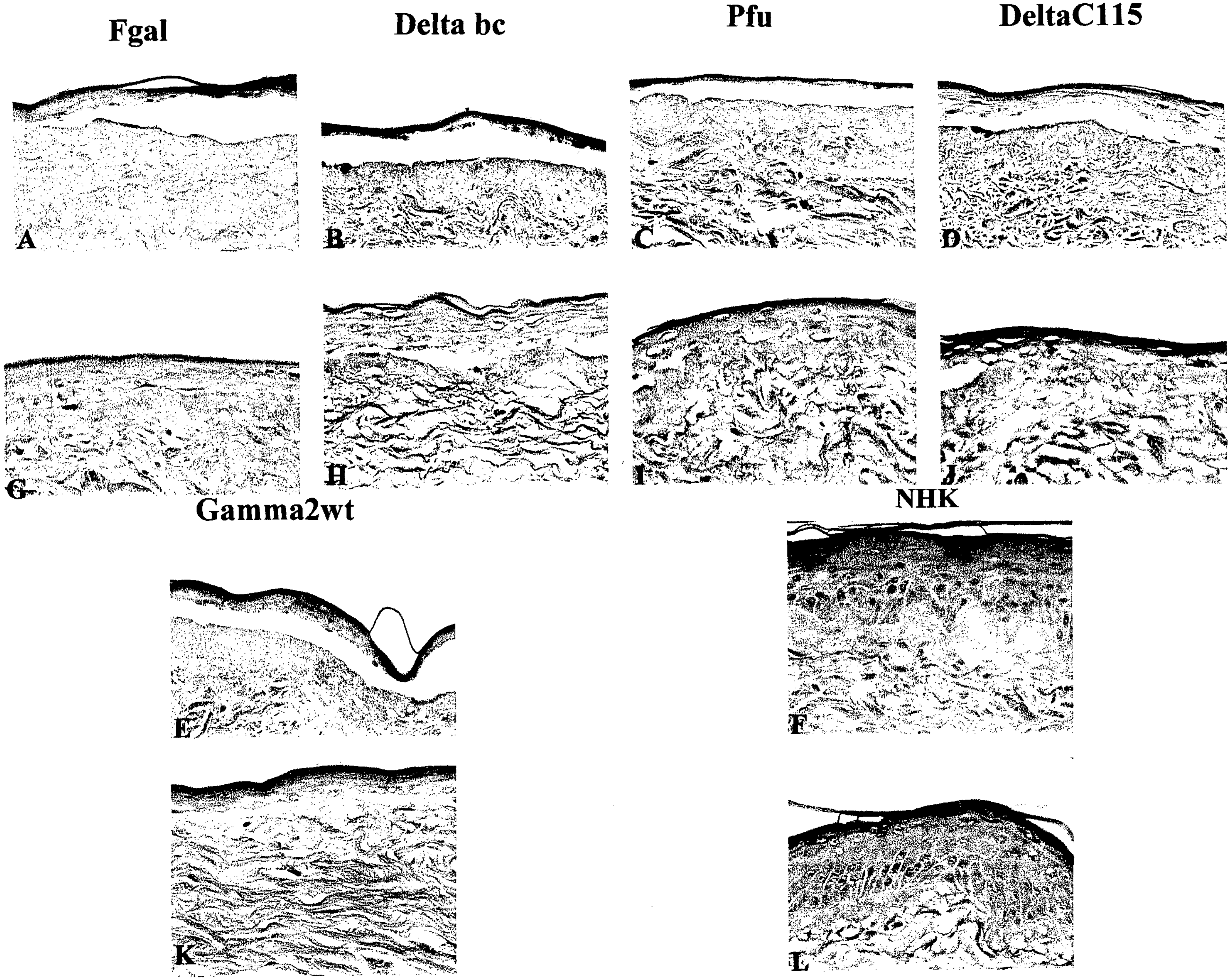
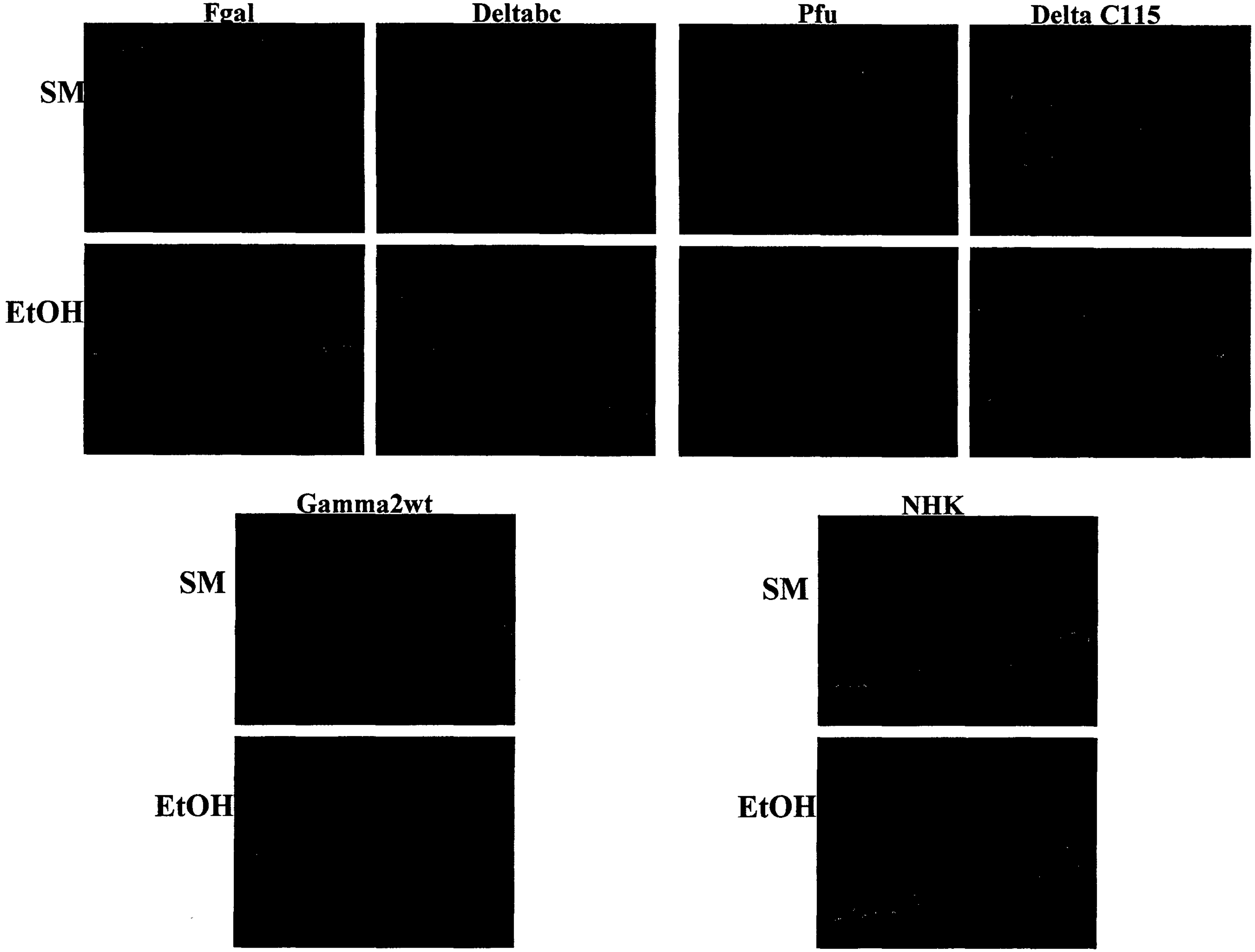



Fig: A-F – 150um SM; G-L- 1% Ethanol.

Fig. 44: M30Staining for diff.cell types on alloderm exposed to SM and Ethanol.




SM—150um; EtOH– 1%

# Fig. 45: Apoptosis for diff. Cells on alloderm exposed to SM and Ethanol

## Table: Percentage of apoptosis for diff.cell types on alloderm

| Chemicals | fgal | Deltabc | pfu | DeltaC115 | Gamma2wt | NHK |
|---|---|---|---|---|---|---|
| SM(150uM) | 20 | 4 | 17 | 7 | 19 | 5 |
| 1%EtOH | 2 | 3 | 4 | 4 | 5 | 2 |